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(54) Title: A METHOD OF EXPRESSING AND SECRETING SOLUBLE EXTRACELLULAR DOMAINS OF HUMAN GONADOTROPIN HORMONE RECEPTORS

(57) Abstract

The extracellular domain, or fragment thereof, of a gonadotropin glycoprotein hormone receptor is expressed and secreted in a soluble and functionally hormone-binding form. A recombinant baculovirus transfer vector is constructed to include a gene segment encoding the extracellular domain, or fragment thereof, of the glycoprotein hormone receptor joined in frame with a gene segment encoding a baculovirus signal peptide and operably linked to a baculovirus promoter. Recombinant baculovirus generated by transfection or co-transfection of insect cells are then used to infect insect host cells for the expression and secretion of soluble receptor.

A METHOD OF EXPRESSING AND SECRETING SOLUBLE EXTRACELLULAR DOMAINS OF HUMAN GONADOTROPIN HORMONE RECEPTORS

BACKGROUND OF THE INVENTION

Field of the Invention

5   The invention relates to a method of generating soluble extracellular domains of human gonadotropin hormone receptors in functional form by expression and secretion in the baculovirus/insect cell system. The invention is also related to a recombinant baculovirus transfer vector carrying

10 DNA encoding the soluble receptor extracellular domains, insect cells expressing and secreting the soluble receptor extracellular domains, and a method of purifying these same soluble receptor extracellular domains.

Description of the Background Art

15   Follicle stimulating hormone (FSH), luteinizing hormone (LH), chorionic gonadotropin (CG), and thyroid stimulating hormone (TSH) are large heterodimeric glycoproteins having a molecular weight of approximately 28-38 kDa and composed of a common α subunit non-covalently bound to

20 a specific β subunit that confers receptor binding specificity. While FSH, LH, and CG are classified as gonadotropins, TSH is not. However, TSH is considered a gonadotropin-like hormone by virtue of sharing many common characteristics with the gonadotropins FSH, LH, and CG. This

25 family of gonadotropin/gonadotropin-like glycoprotein hormones all interact selectively with specific receptors comprising a subclass in the superfamily of G protein-coupled receptors which display seven transmembrane spanning segments as part of their transmembrane domain.

30   Like other known families of G protein-coupled receptors, the glycoprotein hormone receptors have an extracellular domain and a cytoplasmic tail in addition to the distinctive transmembrane domain. However, the glycoprotein hormone receptor family is distinguished from other G protein-

35 coupled receptors, such as rhodopsin, β2-adrenergic receptors, etc., by the large size (>300 amino acid residues) of its extracellular domain or "ectodomain," which also contains multiple cysteine residues and N-linked glycosylation sites.

Upon activation by their respective hormone ligands, the
glycoprotein hormone receptors stimulate an increase in
adenylyl cyclase activity and elevated intracellular adenosine
3', 5'-cyclic phosphate (cAMP) levels (Verrier et al., Eur. J.
5   Biochem., 74:243-252, 1977; Hunzicker-Dunn et al., in
Luteinizing Hormone Action and Receptors, p. 57, 1985, CRC
Press, Boca Raton, FL; Field, in The Thyroid, p. 288, 1986, JB
Lippencott Co., Philadelphia, PA).

          The binding specificity of the glycoprotein hormone
10   receptor and its hormone ligand appears to reside principally
in the extracellular portion of the receptor. In studies with
the LH receptor, variants of the LH receptor molecule lacking
the transmembrane domain and the C-terminal regions have been
found to bind LH with high specificity and affinity (Tsai-
15   Morris et al., J. Biol. Chem. 265:19385-19388, 1990; Xie et
al., J. Biol. Chem. 265:21411-21414, 1991). Binding
specificity has also been found to be conferred by the
extracellular domain in chimeric glycoprotein hormone
receptors created by interchanging N-terminal portions that
20   include the extracellular domain (Braun et al., EMBO J.
10:1885-1890, 1991; Nagayama et al., Biochem. Biophys. Res.
Commun. 173:1150-1156, 1990 and Proc Natl. Acad. Sci. USA
88:902-905, 1991). Davis et al., Mol. Endocrinol. 9:159-170,
(1995) recently reported that the expression of the
25   extracellular domain of rat FSHR by itself is sufficient for
high affinity interactions with human FSH.

          Although the extracellular high affinity hormone-
binding domain of the rat LH receptor has been shown to be
expressed independently of the domain that anchors the
30   receptor in the cell membrane, the expressed extracellular
domain was found to be retained intracellularly in the human
kidney and COS host cells (Xie et al., J. Biol. Chem.
265:21411-21414, 1990; Moyle et al., J. Biol. Chem 266:10807-
10812, 1991; Ji et al., Endocrinology 128:2648-2650, 1991;
35   Pajot-Augy et al., J. Mol. Endocrinol. 14:51-66, 1995) with
the exception of one study (Tsai-Morris et al., J. Biol. Chem.
265:19385-19388, 1990). Tsai-Morris et al., supra, reportedly
found a naturally occurring soluble variant of the LH

receptor, which is believed to be a product of alternative splicing, being secreted extracellularly by COS-7 cells. However, when Moyle et al., supra, expressed this same variant in COS-7 cells, they were unable to reproduce the secretion of

5 soluble modified LH receptor into the culture medium. Instead, they observed that the LHR analogs or variants lacking the transmembrane domain were predominantly retained inside the cell. Kolena et al., Endocrinol. Exp. 20:339-348 (1993) and Wimalasena et al., Endocrinol. 113:618-624 (1983)

10 reported the existence of naturally occurring soluble LH/CG binding proteins which might be receptor fragments. Bernard et al., Mol. Cell. Endocrinol. 71:R19-R23 (1990) reported that alternatively spliced mRNA encoding a truncated LH receptor was expressed in rat ovary which may be an explanation for the

15 previously reported soluble LH receptors. In a separate study with a truncated form (346 N-terminal amino acids) of rat FSHR representing the extracellular domain, it was reported that this truncated receptor was not secreted from human embryonic kidney cells, but rather trapped intracellularly despite the

20 presence of a signal peptide and the absence of any membrane anchoring regions (Davis et al., supra).

           The extracellular domain of the LH receptor was efficiently expressed in E. coli as a truncated receptor which formed inclusion bodies that required refolding with a mild

25 denaturant, guanidine-HCl, to generate a multimeric hormone binding-component soluble form of the receptor (Chen et al., Mol. Cell. Endocrinol. 91:35-41, 1993). As heterologous proteins are expressed in E. coli host cells in nonglycosylated form, the work of Chen and coworkers suggests

30 .that the N-terminal 1-294 amino acid sequence of the LH receptor appears to have all the necessary information required for proper folding and that carbohydrate moieties on the soluble receptor are not required for proper folding or hormone binding activity of the soluble receptor extracellular

35 domain. In contrast, Davis et al., supra, concludes from the results of their studies on N-linked glycosylation of rat FSHR, that while N-linked rat FSHR carbohydrates do not directly provide a binding site for the hormone, they instead

appear to be required for proper folding of nascent receptors
into a hormone-binding competent configuration.

    A number of research groups have tried to take
advantage of the baculovirus/insect cell system to overexpress
5 gonadotropin-like hormone receptors either in full length or
as truncated soluble receptor forms. The baculovirus/insect
cell expression system offers the advantages of having strong
or moderately strong promoters available for the high level
expression of a heterologous protein as well as being
10 noninfectious to vertebrates. This helper virus-independent
expression system has been used to express heterologous
proteins from a variety of different eukaryotes, prokaryotes,
and viruses at levels up to 25% of total insect cell protein.

    Full length rat FSHR and human FSHR were reported to
15 have been expressed in the baculovirus/insect cell system as
functional receptors (Liu et al, Endocrinol. 135:682-691,
1994; Christophe et al., Biochem. Biophys. Res. Commun.
196:402-408, 1993). However, no provisions for secretion of
the soluble extracellular form of the receptor into the
20 culture media was described in either case. Similarly, EP
0614975 A1 relates to the purification and cloning of
receptors for LH, CG, FSH, and TSH but only specifically
discloses examples with rat luteal LH/CG receptor and rat
testicular FSH receptor. Again, no provisions for the
25 secretion of a soluble extracellular form of the receptor was
disclosed.

    Tilly et al. (Society For The Study of Reproduction,
vol. 46, supplement 1, abstract #330, 1992) reported the
expression of a hormone-binding extracellular domain of hFSHR
30 which was retained intracellularly in the soluble cell
fraction of human fetal kidney cells. No secretion of soluble
hFSHR receptor was ever disclosed.

    Seetharamaiah et al. (Autoimmunity 14:315-320, 1993)
disclosed that the hTSHR-EC domain of amino acid residues 1-
35 395, was expressed in a baculovirus/insect cell system and
obtained from whole cell lysates with no secreted receptor
being detected in the culture media as no sequence encoding a
signal peptide was present in the gene construct. In a later

study, Seetharamaiah et al. (Endocrinol. 134:549-554, 1994)
also reported the expression of hTSHR-EC in Sf9 insect cells
where the receptor was obtained as protein aggregates from
whole cell extracts and solubilization with guanidine-HCl was
5 required for proper refolding into a soluble hormone-binding
competent configuration.

In contrast, Huang et al. (J. Mol. Endocrinol.
10:127-142, 1993) reported the expression in a
baculovirus/insect cell system of hTSHR-EC which failed to
10 bind TSH hormone. The gene sequence encoding hTSHR-EC
included the endogenous human signal sequence. Very minute
amounts of truncated receptor, detectable in SDS-PAGE only
when labeled with ^{35}S, were found to be secreted, with almost
all of the hTSHR-EC produced being retained intracellularly.

15   To summarize previous reports describing expression
of extracellular domain of gonadotropin or gonadotropin-like
receptors, the expressed truncated receptor was found to be
trapped intracellularly, frequently in a non-functional form
that requires further purification and refolding with a mild
20 denaturant in order to obtain soluble hormone-binding
competent receptor. Even gene constructs that included a
signal sequence to facilitate secretion in host cells were
unsuccessful in achieving any significant secretion of soluble
receptors, functionally hormone-binding or otherwise.
25 Previously, it has not been possible to reproducibly
demonstrate secretion of any soluble gonadotropin or
gonadotropin-like receptor. A possible explanation for this
failure to secrete soluble hormone-binding receptors may be
due to soluble receptors being identified as misfolded
30 proteins by the cell and retained for degradation (Klauser et
al., Cell 62:611-614 (1990)).

Citation of any document herein is not intended as
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35 present application. Any statement as to content or a date of
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admission as to the correctness of such a statement.

SUMMARY OF THE INVENTION

The present invention provides a method of expressing and secreting the soluble extracellular domain, fragments or variants thereof, of human gonadotropin hormone receptors in a functional hormone-binding form. The baculovirus/insect cell system is advantageously used in generating these soluble receptors.

The method of the present invention overcomes the difficulty of expressing and secreting soluble receptors in a correctly folded and hormone-binding competent form, as previously encountered in the art, by replacing the native human signal sequence with a baculovirus signal sequence capable of promoting correct folding and secretion in insect host cells.

It is therefore an object of the invention to overcome the deficiencies in the prior art by providing a method of expressing and secreting the soluble extracellular domain of gonadotropin hormone receptors.

Another object of the invention is to provide a recombinant baculovirus transfer vector carrying the DNA encoding the extracellular domain of gonadotropin hormone receptors.

A further object of the invention is to provide insect cells infected with recombinant baculovirus and which express and secrete soluble receptor extracellular domains.

Yet another object of the invention is to provide a method of purifying the soluble receptor extracellular domains secreted by insect cells into the culture media.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows the specific binding of CHO-derived soluble hFSHR-EC to ^{125}I-hFSH where the amount of ^{125}I-hFSH bound, expressed as cpm bound, was measured with increasing receptor concentrations (or increasing dilution factors) from left to right along the x-axis.

Figure 2 shows a schematic diagram of the construction of transfer vector pAcGP67-hFSHR-EC where hatched arrow denotes the gene segment encoding the hFSHR-EC.

Figure 3 shows the nucleotide sequence (SEQ ID NO:
1) of the gene segment encoding the hFSHR-EC fused to a signal
sequence and operably linked to a polyhedrin promoter sequence
in the pAcGP67-hFSHR-EC transfer vector of Fig. 1. The amino

5   acid sequence (SEQ ID NO: 2) of the translational fusion of
hFSHR-EC to the gp67 signal peptide is also shown with the
numbering beginning with Met residue of the gp67 signal
peptide. The arrow indicates the presumed cleavage site of
the gp67 signal peptide.

10   Figure 4 shows the specific binding of soluble
hFSHR-EC, secreted from insect cells into the culture medium,
to ^{125}I-hFSH. The amount of ^{125}I-hFSH bound to insect cell-
derived soluble hFSHR-EC, expressed as cpm bound, was measured
with increasing media (receptor) concentrations (or increasing

15 dilution factors) from left to right along the x-axis.

Figure 5 shows a binding assay comparing competitive
displacement of ^{125}I-hFSH from soluble hFSHR-EC derived from
both CHO cell lysates and from baculovirus/insect cell media
by the presence of cold rhFSH. Increasing concentrations of

20 non-radiolabeled rhFSH (from left to right along x-axis)
compete with radiolabeled rhFSH binding to hFSHR-EC.

Figure 6 shows a comparison of specific counts of
^{125}I-FSH bound to soluble receptors produced in previous small
scale batch (FSHR-EC-I) and in a 100 ml scale-up batch (FSHR-

25 EC-II). Media from an abundantly expressed glycoprotein not
related to the gonadotropin receptor generated under the same
conditions as FSHR-EC in baculovirus/insect cells are shown as
a negative control.

Figure 7 shows a UV chromatogram at 280 nm of the

30 0.5 M NaCl washing (fraction E) and elution of hFSHR-EC with
0.1 M acetic acid (fractions J-M) from the FSH-ligand affinity
column.

Figure 8A shows the progesterone levels (y-axis,
ng/ml) in Y1-hFSHR cell medium resulting from treatment with

35 increasing concentration of r-hFSH (open squares) or from a
constant dose of r-hFSH (0.03 nM) plus increasing
concentrations of hFSH-EC from baculovirus/insect cells
culture medium (closed circles). The solutions of r-hFSH and

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hFSHR-EC were made at room temperature and added to the Y1-
hFSHR cells within one hour. The concentration units on the
x-axis for r-hFSH were from amino acid analysis and for hFSHR-
EC were estimates based on binding of hFSHR-EC in culture
5   medium to ^{125}I-hFSH of known specific activity. The highest
concentration of hFSHR-EC tested in this experiment (8.9×10^{-11}M)
was made by diluting the original culture medium by a final
dilution factor (in the assay well) of 0.0223.

Figure 8B shows the progesterone levels (y-axis,
10   ng/ml) in Y1-hFSHR cell medium resulting from treatment with
increasing concentration of r-hFSH (open squares) or from a
constant dose of r-hFSH (0.03 nM) plus increasing
concentrations of hFSH-EC from baculovirus/insect cells
culture medium (closed circles). The solutions of r-hFSH and
15   hFSHR-EC were preincubated at 37°C for one hour and then added
to the Y1-hFSHR cells. The concentration units on the x-axis
are as described for Figure 8A.

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides a method for
20   expressing and secreting the extracellular domain, or fragment
thereof, of a gonadotropin hormone receptor in a soluble and
functional (hormone-binding competent) form. The method first
involves the construction of a recombinant molecule carrying a
gene segment, which encodes the extracellular domain of the
25   gonadotropin hormone receptor joined in translational reading
frame with a gene segment encoding a baculovirus signal
peptide, operably linked to a baculovirus promoter. After
construction of this recombinant molecule, co-transfection
with baculovirus DNA leads to recombinant baculovirus
30   particles which are then used to infect insect host cells to
produce soluble gonadotropin receptors that are secreted into
the culture medium.

The term "gonadotropin", as used herein, for hormone
receptors is intended to include glycoprotein hormone
35   receptors of FSH, LH, and CG that are classified as
gonadotropin receptors and together form a subclass of the G
protein-coupled receptor superfamily.

Preferably, the gonadotropin receptors and the extracellular domain expressed and secreted in the present invention are human receptors which are capable of recognizing and selectively binding their corresponding human glycoprotein
5 hormone. While soluble hormone receptors are useful in diagnostic assays, soluble human glycoprotein receptors, which would not be expected to produce any significant immunological response in humans, can also be used *in vivo* to modulate hormone action in humans.
10 The nucleotide sequences of human testis FSHR (Kelton *et al.*, *Mol. Cell. Endocrin.* 89:141-151, 1992), human ovary FSHR (Minegishi *et al.*, *Biochem. Biophys. Res. Commun.* 175:1125-1130, 1991), and human LH/CG receptor (Minegishi *et al.*, *Biochem. Biophys. Res. Commun.* 172:1049-1054, 1990) are
15 published and available for use in constructing the recombinant molecule for expression and secretion of extracellular domains of these human gonadotropin hormone receptors. It has been shown that the high affinity hormone-binding site resides in the extracellular domain of these
20 gonadotropin receptors.

In expressing the extracellular domain of gonadotropin receptors in insect host cell systems such as *Spodoptera frugiperda* and *Trichoplusia ni*, a baculovirus promoter is used according to the present invention. The
25 baculovirus late promoters (e.g., 39K protein promoter, basic protein promoter) or very late promoters (e.g., p10 promoter, polyhedrin promoter) are preferred for promoting the transcription of an "operably linked" gene sequence encoding a receptor extracellular domain. The polyhedrin and p10
30 promoters, which are expressed during the very late phase of viral infection, are considered very strong promoters capable of expressing large amounts of recombinant protein. While the basic protein promoter and 39K protein promoter are both moderately strong promoters expressed during the late phase of
35 baculovirus expression, they may indirectly provide for better post-translational modification of expressed protein. As more modifying enzymes are present during the late phase of the baculovirus infection cycle than during the very late phase,

the expression from promoters expressed in the late phase of infection may facilitate any post-translational modifications needed.

5   A nucleic acid molecule, such as DNA, is said to be "capable of expressing" a polypeptide, such as the extracellular domain of gonadotropin hormone receptors, if it contains nucleotide sequences which contain transcriptional and translational regulatory information and such sequences are "operably linked" to nucleotide sequences which encode the
10   polypeptide. An operable linkage is a linkage in which the regulatory DNA sequences and the DNA sequence sought to be expressed are connected in such a way as to permit gene expression. The regulatory regions needed for gene expression in general include a promoter region as well as the DNA
15   sequences which, when transcribed into RNA, will signal the initiation of protein synthesis. Such regions will normally include those 5'-non-coding sequences involved with initiation of transcription and translation.

     Two DNA sequences (such as a promoter region
20   sequence and a coding sequence) are said to be operably linked if the nature of the linkage between the two DNA sequences does not (1) result in the introduction of a frame-shift mutation, (2) interfere with the ability of the promoter region sequence to direct the transcription of the coding
25   sequence, or (3) interfere with the ability of the coding sequence to be transcribed by the promoter region sequence. A promoter region would be operably linked to a DNA sequence if the promoter were capable of effecting transcription of that DNA sequence. Thus, to express the protein, transcriptional
30   and translational signals recognized by the host cell are necessary.

     A promoter is a double-stranded DNA or RNA molecule which is capable of binding RNA polymerase and promoting the transcription of an "operably linked" nucleic acid sequence.
35   As used herein, a "promoter sequence" is the sequence of the promoter which is found on the DNA or RNA and is transcribed by the RNA polymerase.

     Table 1 lists examples of baculovirus signal peptide

(leader) sequences or other insect cell signal peptide sequences that have been reported to facilitate the secretion of heterologous proteins in the baculovirus/insect cell expression systems.

Table 1

Signal sequences used for protein expression and secretion in baculovirus/insect cells

Protein(s) expressed	Species of origin, associated protein, and sequence of signal peptide used for expression and secretion.		Reference
HIV-1 gp120	baculovirus ecdysteroid UDPglucosyltransferase (egt)	SEQ ID NO:6	1
	baculovirus surface glycoprotein p67	SEQ ID NO:7	
CD4	Adipokinetic hormone (AKH) from Lepidoptera Manduca sexta	SEQ ID NO:8	2, 3, 4
CD4	Orthoptera Schistocerca gregaria AKH	SEQ ID NO:9	3, 4
	Diptera Drosophila melanogaster (DDM) cuticle protein-1 (CP1)	SEQ ID NO:10	
	DDM CP2	SEQ ID NO:11	
	DDM CP3	SEQ ID NO:12	
	DDM CP4	SEQ ID NO:13	
Plasminogen activator, Urokinase	D. meanogaster α-amylase	SEQ ID NO:14	5
	A. polyhemus chorion A	SEQ ID NO:15	
	H. cecropia cecropin B	SEQ ID NO:16	
		SEQ ID NO:17	
Herpes simplex virus Type 1 glycoprotein gD, Propapain	Apis melifica honeybee melittin	SEQ ID NO:18	6,7

1 Murphy et al., Protein Expression and Purification 4:349-357 (1993).
2 Summers et al., U.S. Patent 5,023,328.
3 Summers, U.S. Patent 5,155,037.
4 Summers, U.S. Patent 5,278,050.
5 Clark et al., WO 90/05783.
6 Tessier et al., Gene 98:177-183 (1991).
7 Sisk et al., J. Virol. 68:766-775 (1994).

According to the method of the present invention, the soluble extracellular domain of gonadotropin hormone receptors are expressed and secreted from the baculovirus/insect host cell system. To effect secretion of

5 the receptor extracellular domain in insect host cells, the extracellular domain is expressed together with a baculovirus signal peptide as a fusion protein. While it is possible that other baculovirus signal or leader sequences may facilitate secretion of significant amounts of receptor extracellular

10 domain, the baculovirus gp67 envelope surface glycoprotein signal peptide from *Autographa californica* is the preferred signal peptide sequence for fusion with a receptor extracellular domain. By ligating DNA encoding for the gp67 signal peptide (SEQ ID NO: 5), to the gene segment encoding

15 for the receptor extracellular domain, a fusion protein can be expressed which allows for a soluble extracellular domain of a gonadotropin hormone receptor to be correctly processed through the insect host cell secretory pathway and secreted into the culture medium in a functional and high affinity

20 hormone-binding form.

The manner in which the signal sequence is ligated to the gene segment encoding for a receptor extracellular domain as well as the manner in which a baculovirus promoter sequence is placed 5' to the signal sequence can be achieved

25 in a variety of ways by those of skill in the art. The ligations may be accomplished with blunt-ended termini or staggered-ended termini having partially or completely complementary staggered ends, or ends created artificially by oligonucleotide primers, such as used for site-specific

30 mutagenesis and PCR amplification, and may be performed in any order depending on the chosen cloning strategy.

Factors to be considered in constructing an operably linked promoter-signal sequence-gene fragment of interest as a contiguous segment for expression in insect cells include

35   (1) keeping the 5' untranslated sequence between the promoter sequence and the ATG translational initiation codon to a minimum, with up to approximately 100 nucleotides being generally acceptable;

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(2) being careful not to introduce any additional ATG codon, whether out of frame or in frame with the coding sequence, that is 5' to the coding sequence for the gene of interest, as translation normally initiates from the first ATG

5 codon encountered 3' of the baculovirus promoter;

(3) whether or not to create additional amino acid residues or delete amino acid residues at the N-terminus of the receptor extracellular domain by the strategy used to join the signal sequence to the gene fragment of interest.

10 Variants of the extracellular domain of gonadotropin receptors are included in the soluble receptors to be expressed and secreted by the method of the present invention, some of which can be obtained by modifying the sequence at the junction between the signal sequence and the gene fragment of

15 interest.

In order to introduce the operably linked promoter-signal sequence-gene fragment of interest construct into insect cells and generate soluble gonadotropin hormone receptors that are secreted into the culture medium, the

20 entire construct must be part of a baculovirus transfer vector. Insect cells can be transfected or co-transfected to produce recombinant baculoviruses. In the case of transfection, a baculovirus transfer vector may be recombined with another vector, such as a baculovirus shuttle vector, or

25 "bacmid", etc., and the resultant recombinant baculovirus shuttle vector containing the promoter-signal sequence-gene fragment of interest can then be isolated for transfection into insect cells. The BAC-TO-BAC™ expression system available from GIBCO BRL, Gaithersburg, MD, is an example of

30 using transfection alone to introduce a recombinant baculovirus shuttle vector containing the promoter-signal sequence-gene fragment of interest as an expression cassette. In co-transfection, the baculovirus transfer vector, containing sequence homology to baculovirus DNA, allows for

35 homologous recombination with the co-transfected baculovirus DNA and recombinant baculoviruses can then be either screened or selected.

Additionally, a baculovirus transfer vector may

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advantageously carry a viral or bacterial origin of
replication to allow for amplification of the vector construct
in bacteria prior to co-transfection of insect host cells.

5   It will be understood that co-transfection of the
baculovirus transfer vector (carrying the soluble receptor
construct) with baculovirus DNA provides a means of generating
recombinant baculoviruses by allowing for homologous
recombination between the baculovirus DNA and the baculovirus
transfer vector. Therefore, the baculovirus transfer vector
10   used in co-transfection with baculovirus DNA would necessarily
carry a region homologous with baculovirus DNA, such as the
polyhedrin locus. To improve recombination efficiency, a
modified baculovirus DNA carrying a polyhedrin promoter-driven
lacZ gene coding for β-galactosidase can be linearized at a
15   single site with a restriction enzyme and recombinants can be
screened by their color on X-gal plates where non-recombinants
are stained blue (lac Z-positive) and recombinants remain
unstained (lac Z-negative) (see AcRP23.lacZ and AcUW1.lacZ
baculovirus DNA, Catalogue #21101D and #21102D, respectively,
20   Pharmingen, San Diego, CA).

       However, it is preferred that the baculovirus
transfer vector carry an essential baculovirus gene which can
complement a lethal deletion in a modified baculovirus DNA.
In this way, only recombinant baculoviruses, where the
25   recombination event has rescued the region complementing the
lethal deletion, are selected. A preferred baculovirus
transfer vector and modified baculovirus DNA combination is
the pAcGP67-A, -B, or -C set of transfer vectors with
BACULOGOLD baculovirus DNA, both available from Pharmingen
30   (San Diego, CA). BACULOGOLD carries a lethal deletion of an
essential gene coded for by the open reading frame 1629 (ORF
1629) which can be rescued by baculovirus transfer vectors
carrying ORF-1629 normally located in a region spanning 1.7 Kb
downstream from the stop codon of the polyhedrin gene.

35   Standard reference works setting forth the general
principles of recombinant DNA technology include Ausubel et
al., eds., Current Protocols In Molecular Biology, Green
Publishing Assoc. and Wiley Interscience, N.Y. (1987-1994),

Watson et al., *Molecular Biology of the Gene*, Volumes I and II, The Benjamin/Cummings Publishing Company, Inc., publisher, Menlo Park, CA (1987); Darnell et al., *Molecular Cell Biology*, Scientific American Books, Inc., publisher, New York, N.Y.
5 (1986); Lewin, *Genes II*, John Wiley & Sons, publishers, New York, N.Y. (1985); Old et al., *Principles of Gene Manipulation: An Introduction to Genetic Engineering*, 2d edition, University of California Press, publisher, Berkeley, CA (1981); Sambrook et al., *Molecular Cloning: A Laboratory*
10 *Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY (1989); and Berger et al., *Guide to Molecular Cloning Techniques*, Methods of Enzymology vol. 152, Academic Press, Inc., publisher, San Diego, CA, (1987). These references are hereby entirely incorporated by reference.
15   A number of well-established insect cell lines that are susceptible to baculovirus infection, such as cell lines of *Spodoptera frugiperda* and *Trichoplusia ni*, are readily available. For instance, the *Trichoplusia ni* HI-FIVE (BTI-TN-5B1-4) cell line can be obtained from Invitrogen (San Diego,
20 CA) and *Spodoptera frugiperda* cell lines Sf9 and Sf21, established from ovarial tissues of *S. frugiperda* larvae, can be obtained from Invitrogen or Pharmingen (San Diego, CA), ATCC (Catalogue #CRL 1711) among others. In addition, live insect larvae can also be used as the host cell system.
25   Generally, a first insect cell line is used to generate recombinant baculovirus for viral stock by co-transfection and a second insect cell line is later infected with virus from this recombinant baculovirus stock for the production of soluble gonadotropin-like receptors. The first
30 and second insect cell line used according to the method of the present invention may be the same cell line or may be two different cell lines. In a preferred embodiment, the first insect cell line, that is co-transfected to generate recombinant baculovirus is *Spodoptera frugiperda* Sf9 cell line
35 and the second insect cell line for soluble receptor production is *Trichoplusia ni* HI-FIVE cell line.
       Protocols for handling of insect cell lines including culturing, co-transfection, assays for viral titer,

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virus amplification, infection of insect cells, etc., as well
as teachings on promoter, vector/DNA, etc., consideration and
selection are set forth in Gruenwald et al., *Baculovirus
Expression Vector System: Procedures and Methods*, 2nd ed.,

5   1993, Pharmingen, San Diego, CA; O'Reilly et al., *Baculovirus
Expression Vectors, A Laboratory Manual*, W.H. Freeman and Co.,
publisher, New York, 1992; Ausubel et al., *supra*, among other
standard reference works in the art. The above references are
herein entirely incorporated by reference.

10   The soluble hormone-binding extracellular domains of
gonadotropin hormone receptors expressed and secreted in
accordance with the present method can be linked to an
affinity column to extract hormone mimetics or antagonists
from heterogeneous mixtures such as natural fluids or chemical

15   libraries, and to purify gonadotropin hormones from fluids,
extracts, etc. The purified gonadotropin hormones can then be
used as therapeutics in the treatment of infertility (FSH),
etc.

                Like the purified gonadotropin hormone, the soluble

20   hormone-binding extracellular domains can also be used
therapeutically, such as to selectively modulate hormone
action *in vivo*. For example, hLH/CGR-EC can be used as a
contraceptive agent, with or without coupling to a
nonproteinaceous polymer in order to increase the biological

25   half-life of the soluble receptor molecule. Soluble hFSH
receptors produced and secreted according to the method of the
present invention were found to antagonize FSH binding and
stimulation in an *in vitro* live cell assay. Increasing
amounts of added soluble hFSH receptor were observed to block

30   cell stimulation with FSH as monitored by the level of
progesterone produced in the live cell assay.

                Soluble receptors can also be used in place of
monoclonal antibodies to provide solution-based radioligand
receptor assays which would detect only receptor-binding

35   competent hormones with virtually no cross-reactivity to free
hormone subunits. Similarly, antibody-receptor sandwich
assays or enzymatic assays can be performed with the soluble
receptor EC domain generated by the method of the present

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invention.

As the gonadotropin hormones, FSH, LH, and CG bind
to their respective glycoprotein hormone receptors with high
affinity and specificity, these characteristics make soluble
5 hormone-binding receptors attractive reagents for screening
candidate agonists or antagonists.

Other possible diagnostic and therapeutic utilities
for which soluble gonadotropin-like hormone receptors would be
useful are described in detail in EP 0614975 A1, the contents
10 of which are incorporated herein by reference.

Essentially pure extracellular domains of
gonadotropin hormone receptors can also be used in x-ray
crystallographic analysis to develop molecular models which
define the tertiary structure of the hormone-binding domains.
15 Such information would provide insight into the structure of
the actual contact between a hormone and its receptor.
Structural information of this sort would be useful in the
design of peptides which have gonadotropin-like agonistic or
antagonistic activity.

20 The extracellular domain of gonadotropin receptors
to be expressed and secreted according to the method of the
present invention can be the entire extracellular domain of
these receptors, e.g., amino acid residues 1-349 of the mature
hFSHR amino acid sequence beginning with Cys-His-His, variants
25 thereof or fragments/portions of the extracellular domain.
Since it appears that the complete extracellular domain is not
necessary to bind the corresponding hormone with high
affinity, it is contemplated that a fragment or portion of the
amino acid terminal extracellular domain may be effectively
30 utilized as a soluble receptor.

Using hFSHR as an example, a fragment which is
somewhat shorter than the 349 amino acids of the complete
extracellular domain may be readily produced and tested for
effective binding to FSH. So long as the hormone-binding
35 region of the extracellular portion is maintained intact, the
length of the overall fragment utilized is not critical. For
this reason, it is also expected that non-interfering amino
acids can be added to either end of the extracellular domain,

or hormone-binding fragment thereof, without adversely
affecting the hormone-binding capacity of the polypeptide.
Accordingly, this invention includes a fragment of the human
gonadotropin hormone receptor which comprises a substantial

5 portion of the extracellular domain and which retains
substantially the same hormone-binding characteristics as the
complete extracellular domain.

        Variants or mutant forms of the extracellular
domain, or fragment thereof, of the gonadotropin receptors are

10 also intended to be included as the soluble receptors to be
expressed and secreted by the method of the present invention.
Such variants are those where at least one amino acid residue
in the polypeptide has been conservatively replaced by a
different amino acid, the location and nature of such

15 substitutions being selected so as not to significantly affect
the hormone-binding characteristics of the soluble receptor or
fragment thereof which is modified. Thus, the amino acid or
nucleic acid sequence of a variant is said to "essentially
correspond" to another amino acid or nucleic acid sequence,

20 respectively, if the sequence of amino acids or nucleic acid
in both molecules provides polypeptides having biological
activity that is essentially similar, qualitatively or
quantitatively, to the corresponding polypeptide. Such
"essentially corresponding" hormone receptor extracellular

25 domain sequences include conservative amino acid or nucleotide
substitutions, or degenerate nucleotide codon substitutions
where individual amino acid or nucleotide substitutions are
well known in the art.

        Accordingly, hormone-binding extracellular domains,

30 fragments thereof, or nucleic acid encoding therefor, include
a finite set of essentially corresponding sequences as
substitution peptides or polynucleotides which can be
routinely obtained by one of ordinary skill in the art,
without undue experimentation, based on the teachings and

35 guidance presented herein. For a detailed description of
protein chemistry and structure, see Schulz et al., *Principles
of Protein Structure*, Springer-Verlag, New York, 1978, and
Creighton, *Proteins: Structure and Molecular Properties*, W.H.

Freeman & Co., San Francisco, 1983, which are hereby
incorporated by reference. For a presentation of nucleotide
sequence substitutions, such as codon preferences, see Ausubel
et al, eds, *Current Protocols in Molecular Biology*, Greene
5 Publishing Assoc., N.Y. (1987, 1988, 1989, 1990, 1991, 1992,
1993, 1994) at §§ A.1.1-A.1.24, and Sambrook *et al*, *Molecular
Cloning: A Laboratory Manual*, Second Edition, Cold Spring
Harbor Press, Cold Spring Harbor, NY (1989), at Appendices C
and D.

10   As mentioned previously, conservative substitutions
include variants wherein at least one amino acid residue in
the polypeptide has been conservatively replaced by a
different amino acid. Such substitutions preferably are made
in accordance with the following list as presented in Table 2,
15 which substitutions can be determined by routine
experimentation to provide modified structural and functional
properties of a synthesized or recombinant polypeptide
molecule, while maintaining hormone-binding activity, as
determined by radioligand receptor assays, Scatchard analysis,
20 etc. In the context of the present invention, the term
"essentially corresponding to" includes such substitutions.

Table 2

Original Residue	Exemplary Substitution		
Ala	Gly; Ser		
Arg	Lys		
Asn	Gln; His		
Asp	Glu		
Cys	Ser		
Gln	Asn		
Glu	Asp		
Gly	Ala; Pro		
His	Asn; Gln		
Ile	Leu; Val		
Leu	Ile; Val		
Lys	Arg; Gln; Glu		
Met	Leu; Tyr; Ile		
Phe	Met; Leu; Tyr		
Ser	Thr		
Thr	Ser		
Trp	Tyr		
Tyr	Trp; Phe		
Val	Ile; Leu		

     Accordingly, based on the above example of specific
45 substitutions, alternative substitutions can be made by
routine experimentation, to provide alternative extracellular

domains of gonadotropin receptors, e.g., by making one or more
conservative substitutions. Preferably, even such
conservative substitutions should be in the non-conserved
portions of the extracellular domain. It would be expected
5   that any such substitutions would retain hormone-binding
activity, which activity can be checked with routine
experimentation, as will be described below.

      Alternatively, another group of substitutions are
those in which at least one amino acid residue in the protein
10 molecule has been removed and a different residue inserted in
its place according to the following Table 3. The types of
substitutions which can be made in the protein or peptide
molecule can be based on analysis of the frequencies of amino
acid changes between a homologous protein of different species
15 or conserved amino acids between the human and other mammalian
gonadotropin-like hormone receptors. Based on such an
analysis, alternative conservative substitutions are defined
herein as exchanges within one of the following five groups:

TABLE 3

20   1.   Small aliphatic, nonpolar or slightly polar residues:
          Ala, Ser, Thr (Pro, Gly);

     2.   Polar, negatively charged residues and their amides:
          Asp, Asn, Glu, Gln;

25   3.   Polar, positively charged residues:
          His, Arg, Lys;

     4.   Large aliphatic, nonpolar residues:
          Met, Leu, Ile, Val, (Cys); and

     5.   Large aromatic residues:
          Phe, Tyr, Trp.

30   The three amino acid residues in parentheses above
have special roles in protein architecture. Gly is the only
residue lacking any side chain and thus imparts flexibility to
the chain. This, however, tends to promote the formation of
secondary structure other than α-helical. Pro, because of its
35 unusual geometry, tightly constrains the chain. It generally
tends to promote β-turn-like structures. In some cases Cys
can be capable of participating in disulfide bond formation
which is important in protein folding. Note that Schulz et
al. would merge Groups 1 and 2 above. Note also that Tyr,

because of its hydrogen bonding potential, has significant
kinship with Ser, and Thr, etc. Knowledge of the secondary
structure and of the tertiary structure will assist those of
skill in the art in determining which such substitutions would
5 not be expected to affect the binding capability of the
peptide.

Conservative amino acid substitutions, included in
the term "essentially corresponding", according to the present
invention, e.g., as presented above, are well known in the art
10 and would be expected to maintain the binding properties of
the polypeptide after amino acid substitution. Most deletions
and insertions, and substitutions according to the present
invention are those which do not produce radical changes in
the characteristics of the protein or peptide molecule.
15 "Characteristics" is defined in a non-inclusive manner to
define both changes in secondary structure, e.g., α-helix or
β-sheet, as well as changes in binding activity.

However, when the exact effect of the substitution,
deletion, or insertion is to be confirmed, one skilled in the
20 art will appreciate that the effect of the substitution or
substitutions will be evaluated by routine screening assays,
such as radioligand receptor assays, ELISA, etc., to confirm
biological activity, i.e., hormone-binding.

Most deletions and insertions, and substitutions of
25 the extracellular domain of gonadotropin receptors are those
which maintain or improve the hormone-binding characteristics
of the protein or peptide molecule. However, when it is
difficult to predict the exact effect of the substitution,
deletion, or insertion in advance of doing so, one skilled in
30 the art will appreciate that the effect will be evaluated by
routine screening assays. For example, a variant made by
site-specific mutagenesis of the peptide molecule- encoding
nucleic acid and expression of the variant extracellular
domain in cell culture can be tested for binding by binding
35 assays disclosed herein, to test gonadotropin hormone-binding
capability.

Amino acid sequence variants of the extracellular
domain of gonadotropin receptors can be prepared by mutations

in the DNA.   Such variants include, for example, deletions
from, or insertions or substitutions of, residues within the
amino acid sequence.   Any combination of deletion, insertion,
and substitution can also be made to arrive at expressing and
5   producing the final extracellular domain construct, provided
that the final construct possesses some hormone-binding
activity.   Preferably, improved hormone-binding activity is
found over that of the non-variant peptide.   Obviously, the
mutations that will be made in the DNA encoding the variant
10   must not place the sequence out of reading frame and
preferably will not create complementary regions that could
produce secondary mRNA structure (see, e.g., EP Patent
Application Publication No. 75,444, Ausubel et al., supra,
1987-1994; Sambrook et al., supra, 1989).
15   Amino acid sequence insertions include amino and/or
carboxyl-terminal fusions of from one residue to polypeptides
of essentially unrestricted length, as well as intrasequence
insertions of single or multiple amino acid residues.
Intrasequence insertions may range generally from about 1 to
20   10 residues, more preferably 1 to 5.   An example of a terminal
insertion includes a fusion of a signal sequence, whether
heterologous or homologous to the host cell, to the N-terminus
of the receptor extracellular domain to facilitate secretion
from recombinant hosts.
25   At the genetic level, these variants ordinarily are
prepared by site-directed mutagenesis of nucleotides in the
DNA encoding the extracellular domain of a gonadotropin
receptor, thereby producing DNA encoding the variant, and
thereafter synthesizing the DNA and expressing the peptide in
30   recombinant cell culture.   The variants typically exhibit the
same qualitative biological activity as the naturally
occurring analogue, Ausubel et al., supra, 1987-1994; Sambrook
et al., supra, 1989.
           Preparation of an extracellular domain variant in
35   accordance herewith, or an alternative nucleotide sequence
encoding the same polypeptide but differing from the natural
sequence due to changes permitted by the known degeneracy of
the genetic code, can be achieved by site-specific mutagenesis

of DNA that encodes an earlier prepared variant or a
nonvariant version of the receptor extracellular domain.
Site-specific mutagenesis allows the production of variants
through the use of specific oligonucleotide sequences that

5   encode the DNA sequence of the desired mutation, as well as a
sufficient number of adjacent nucleotides, to provide a primer
sequence of sufficient size and sequence complexity to form a
stable duplex on both sides of the deletion junction being
traversed. Typically, a primer of about 20 to 25 nucleotides

10 in length is preferred, with about 5 to 10 complementing
nucleotides on each side of the sequence being altered. In
general, the technique of site-specific mutagenesis is well
known in the art, as exemplified by publications such as
Adelman et al., DNA 2:183 (1983), the disclosure of which is

15 incorporated herein by reference.

         As will be appreciated, the site-specific
mutagenesis technique typically employs a phage vector that
exists in both a single-stranded and double-stranded form.
Typical vectors useful in site-directed mutagenesis include

20 vectors such as the M13 phage, for example, as disclosed by
Messing et al., Third Cleveland Symposium on Macromolecules
and Recombinant DNA, Editor A. Walton, Elsevier, Amsterdam
(1981), the disclosure of which is incorporated herein by
reference. These phage are readily available commercially and

25 their use is generally well known to those skilled in the art.
Alternatively, plasmid vectors that contain a single-stranded
phage origin of replication (Veira et al., Meth. Enzymol.
153:3 (1987)) may be employed to obtain single-stranded DNA.

         In general, site-directed mutagenesis in accordance

30 herewith is performed by first obtaining a single-stranded
vector that includes within its sequence a DNA sequence that
encodes the relevant protein. An oligonucleotide primer
bearing the desired mutated sequence is prepared synthetically
by automated DNA/oligonucleotide synthesis. This primer is

35 then annealed with the single-stranded protein-sequence-
containing vector, and subjected to DNA-polymerizing enzymes
such as E. coli polymerase I Klenow fragment, to complete the
synthesis of the mutation-bearing strand. Thus, a mutated

25

sequence and the second strand bears the desired mutation.
This heteroduplex vector is then used to transform appropriate
cells, such as *E. coli* JM101 cells, and clones are selected
that include recombinant vectors bearing the mutated sequence
5   arrangement.

After such a clone is selected, the mutated receptor
extracellular domain region may be removed and placed in an
appropriate vector, generally a transfer or expression vector
of the type that may be employed for transfection of an
10   appropriate host.

As previously mentioned above, the nucleotide and
amino acid sequences of human gonadotropin receptors as well
as a number of gonadotropin receptors from other mammalian
species have been published and are readily available to those
15   in the art.

Accordingly, gene or nucleic acid encoding for a
gonadotropin hormone receptor having an extracellular domain
can also be detected, obtained and/or modified, *in vitro*, *in
situ* and/or *in vivo*, by the use of known DNA or RNA
20   amplification techniques, such as PCR and chemical
oligonucleotide synthesis. PCR allows for the amplification
(increase in number) of specific DNA sequences by repeated DNA
polymerase reactions. This reaction can be used as a
replacement for cloning; all that is required is a knowledge
25   of the nucleic acid sequence. In order to carry out PCR,
primers are designed which are complementary to the sequence
of interest. The primers are then generated by automated DNA
synthesis. Because primers can be designed to hybridize to
any part of the gene, conditions can be created such that
30   mismatches in complementary base pairing can be tolerated.
Amplification of these mismatched regions can lead to the
synthesis of a mutagenized product resulting in the generation
of a peptide with new properties (i.e., site directed
mutagenesis). See also, e.g., Ausubel, *supra*, Ch. 16. Also,
35   by coupling complementary DNA (cDNA) synthesis, using reverse
transcriptase, with PCR, RNA can be used as the starting
material for the synthesis of the extracellular domain of a
gonadotropin receptor without cloning.

Furthermore, PCR primers can be designed to
incorporate new restriction sites or other features such as
termination codons at the ends of the gene segment to be
amplified. This placement of restriction sites at the 5' and
5 3' ends of the amplified gene sequence allows for gene
segments encoding an extracellular domain or fragment thereof
to be custom designed for ligation with signal sequences
and/or cloning sites in vectors.

        PCR and other methods of amplification of RNA and/or
10 DNA are well known in the art and can be used according to the
present invention without undue experimentation, based on the
teaching and guidance presented herein. Known methods of DNA
or RNA amplification include, but are not limited to
polymerase chain reaction (PCR) and related amplification
15 processes (see, e.g., U.S. patent Nos. 4,683,195, 4,683,202,
4,800,159, 4,965,188, to Mullis et al.; 4,795,699 and
4,921,794 to Tabor et al.; 5,142,033 to Innis; 5,122,464 to
Wilson et al.; 5,091,310 to Innis; 5,066,584 to Gyllensten et
al.; 4,889,818 to Gelfand et al.; 4,994,370 to Silver et al.;
20 4,766,067 to Biswas; 4,656,134 to Ringold; and Innis et al.,
eds., PCR Protocols: A Guide to Method and Applications) and
RNA mediated amplification which uses anti-sense RNA to the
target sequence as a template for double stranded DNA
synthesis (U.S. patent No. 5,130,238 to Malek et al., with the
25 tradename NASBA); and immuno-PCR which combines the use of DNA
amplification with antibody labeling (Ruzicka et al., Science
260:487 (1993); Sano et al., Science 258:120 (1992); Sano et
al., Biotechniques 9:1378 (1991)), the entire contents of
which patents and reference are entirely incorporated herein
30 by reference.

        Having now generally described the invention, the
same will be more readily understood through reference to the
following examples which are provided by way of illustration
and is not intended to be limiting of the present invention.

Example 1:

Expression and Purification of A Truncated hFSH Receptor
Encompassing the Entire hFSHR-EC Domain in CHO-DUKX Cells

A human FSHR mutant cDNA, designated FSHR366, was

5   constructed to encompass the whole extracellular domain of
full-length hFSHR beginning with Met_1 of the human signal
peptide and truncating at amino acid residue Arg_{366}.
Additionally, the hFSH truncated receptor also contained a six
polyhistidine tail attached to the C-terminal Arg_{366} residue.

10 The DNA fragments encoding hFSHR366 was inserted into an
expression vector carrying DHFR which was then used to
transfect CHO-DUKX cells. Methotrexate resistent clones were
obtained and cultured for analysis of truncated hFSHR
expression and for the purification of truncated receptor.

15   ^{125}I-FSH specific binding was determined for the cell
pellet and cell lysate fractions as well as for the culture
media and intact cells. For each sample, the total binding to
^{125}I-FSH (human) was determined by incubating an aliquot of the
test sample with an aliquot of ^{125}I-FSH at 37°C. The non-

20 specific binding was determined by adding an excess of cold
FSH to an identical tube set up for each sample. The specific
^{125}I-FSH bound radioactive counts in cpm for each sample was
calculated by taking the difference between the counts for the
total bound ^{125}I-FSH and the non-specific bound counts.

25   For the determination of specific FSH binding in
cell pellet and cell lysate fractions, $1-5 \times 10^7$ CHO cells were
suspended in 1 ml of 1% Triton in sucrose/Hepes/Mg/Ca buffer
and then homogenized on dounce. The suspension was diluted
with 9 ml of sucrose/Hepes/Mg/Ca buffer and then spun at

30 10,000 X g for 20 minutes at 4°C. Aliquots of the supernatant
were serial diluted and used directly in the binding assay.
For the binding assay, 90 ul of the CHO-366 supernatant
sample, 10 ul (96,000 cpm, 1.1 nM) of ^{125}I-hFSH, and 10 ul of
H_2O or 1.2 uM rhFSH were combined in Falcon 12x75 mm

35 polypropylene tubes. The solution was incubated at 37°C for
0.75 hours. The solution was placed on ice and 50 ul of 0.5%
(w/v) γ-globulin buffer (in 50 mM Tris, pH 7.35) was added.
After 5 minutes, 160 ul of 21% (w/v) PEG-8000 solution (in 50

mM Tris, pH 7.25) was added to each sample. The samples sat
on ice for approximately 1 hour. The precipitate was pelleted
by spinning at 3750 rpm (ca. 3000 Xg). To bring non-specific
binding to less than 15% of total binding, a second
5   precipitation was carried out. The supernatant was aspirated,
and the pellet was redissolved in 1 ml of 0.1% (v/v) Triton X-
100 in PBS, diluted with 0.5 ml of 0.5% γ-globulin buffer
followed by 1.5 ml of PEG-8000 solution. The mixture stood on
ice for 30 minutes, then centrifuged at 3750 rpm (ca. 3000
10 Xg), and the supernatant was aspirated. Pellets were counted
on a γ-counter. The specific binding of CHO-derived soluble
FSHR-EC to ^{125}I-hFSHR is shown in Figure 1.

The specific binding resulting from cell pellet,
cell lysate, culture medium, and intact cells demonstrate that
15 hFSHR366 is expressed in CHO cells as a soluble receptor which
is found predominantly in the cell lysate or to a much lesser
extent in the cell pellet. Insignificant levels of specific
^{125}I-hFSH binding was observed in the culture medium and intact
cell samples. These results indicate that hFSHR366 is not
20 secreted into the culture medium or present on the surface of
intact whole cells in significant amounts but rather is
trapped intracellularly in CHO cells.

Membrane fractions from cells expressing full-length
hFSHR and cell lysates from cells expressing soluble hFSHR366
25 were processed for Scatchard analysis to measure the FSH
binding affinity of soluble and full-length hFSHR. Scatchard
analysis was determined by adding increasing amounts of cold
FSH to a constant amount of ^{125}I-FSH and a constant amount of
experimental cell sample fraction. The assay contained 30 μl
30 of cell lysate sample or 10 μl of membrane, approximately 1.2
ng ^{125}I-FSH and unlabeled hFSH (in the range of 0 to 4000 ng).
The assay was incubated at 4°C, for either 6 hours, or 16-18
hours at which time equilibrium was reached. For Scatchard
analysis the affinity constant was the slope of the best-fit
35 line through a plot of the concentration of bound hormone
divided by free hormone ([B]/[F]) versus bound hormone ([B]).
[B] and [F] were calculated from the specifically bound counts
measured in the pellet, the total amounts of r-hFSH and ^{125}I-FSH

added to the reaction, and the known specific activity of the
^{125}I-FSH following procedures known by those skilled in the art,
with the aid of an Excel 4.0 program (Microsoft).

        Several anti-peptide human FSHR antibodies were
5 tested for specificity to detect the soluble FSHR in an
antibody capture assay. Polyclonal antibodies W970, W954, and
X179 were obtained from Dr. J. Dias at the Wadsworth Center
for Laboratories and Research, New York (Liu et al.,
Endocrinology 135:682-691, 1994) where: (1) W970 was raised in
10 rabbits immunized with peptides containing rat FSHR amino acid
residues 150 to 183 coupled to KLH; (2) W954 was raised in
rabbits immunized with peptides containing human FSHR amino
acid residues 265 to 296; (3) X179 was similarly raised in
rabbits immunized with human FSHR amino acid residues 265 to
15 296 and coupled at the C-terminus to ovalbumin.

        For the antibody capture assay, medium binding plate
(Catalogue #2586 from Costar) was coated with 5 µg/ml of PBS
diluted cell lysate and left overnight at 4°C. The plate was
aspirated and blocked with 5% BSA for 1 hour at 37°C. Primary
20 antibody W970 at 1:500 dilution was added for 1 hour at 37°C.
The plate was aspirated and washed with 1% BSA in PBS.
Secondary goat anti-rabbit antibody conjugated with
horseradish peroxidase at 1:4000 dilution was incubated for 1
hour at 37°C. The plate was aspirated and washed 3 times with
25 1% BSA in PBS. Hydrogen peroxidase developing solution was
added and left at room temperature for 20 minutes. The plate
was read at 450 nm. All three antibodies showed up to a two-
fold increase in absorbance with the hFSHR366 lysate compared
with that of the parental CHO-DUKX. This indicates that
30 polyclonal antibodies W970, W955/954, and X179 can be used to
detect truncated human FSHR.

        The hFSHR truncated receptor was engineered to
include a hexahistidyl tail (6xHis) at the carboxy terminus in
order to permit affinity purification using an immobilized
35 metal affinity column (IMAC). The column contains nickel
bound through chelation with resin-bound nitrilo-tri-acetic
acid (Ni-NTA). Four of the six chelation sites serve to bind
the nickel to the column while leaving two sites available to

chelate with the 6xHis. Elution is achieved either by
reducing the pH, or adding imidazole. Acid pH protonates the
histidine, causing dissociation from the Ni-NTA. Imidazole
competes with histidine for the Ni-NTA, again causing
5   dissociation of the protein from the resin. The effectiveness
of the washing and elution on Ni-NTA was monitored by SDS-PAGE.
analysis and Coomassie, silver, and immuno-staining were
utilized for visualization of proteins.
            The results obtained from the washing and elution
10   fractions on SDS-PAGE clearly demonstrated that Ni-NTA
partially purified the soluble receptor. Negligible amounts
of soluble receptor was observed in unbound, washed, or 30 mM
imidazole fractions (elution of receptor is with 100 mM
imidazole) by immunostaining with rabbit antibody W970 and a
15   goat anti-rabbit horseradish peroxidase conjugate. Coomassie
staining of these same fractions on SDS-PAGE gels showed many
non-specific proteins being eluted with the washes. Even with
removal of non-specific proteins in the wash fractions, many
other contaminating proteins were still found to be present in
20   the 100 mM imidazole elution fraction.
            In addition to the Ni-NTA column, an affinity resin
was prepared with immobilized FSH. The FSH was covalently
bound to the CNBr resin through primary amines on the protein.
In order to prevent destruction of the column by subunit
25   dissociation under harsh washing or elution conditions, a
method was developed for crosslinking the alpha and beta
subunits of FSH before immobilization on the resin.
Crosslinking presumably occurs between primary amines and
carboxyl groups which are in close proximity, such as in the
30   non-covalent salt bridge potentially involved in heterodimer
association. The crosslinked material was then separated from
both the reagents and the intermolecular crosslinked FSH by
gel filtration chromatography, and the effectiveness of the
crosslinking was determined by SDS-PAGE under conditions that
35   would dissociate the non-crosslinked material. Purified
crosslinked material was then anchored to CNBr resin for use
in purifying the receptor.
            Previously Ni-NTA enriched receptor was applied to

the crosslinked-FSH affinity column to further enrich and
purify the soluble hFSH receptor. The results of elution from
the crosslinked-FSH affinity column indicate that
contaminating proteins are still eluted with the receptor and
5 further purification is needed to obtain a purified soluble
hFSH receptor.

Example 2:

Construction of the Transfer Vector for hFSHR-EC

The hFSHR cDNA, previously described as a 2.1 kb
10 full length hFSHR construct in pUC18 in Kelton *et al.*, *Mol.*
Cell. Endocrinol. 89:141-151 (1992) was amplified by
polymerase chain reaction (PCR) to generate BclI and PstI
restriction sites at the 5' and 3' ends, respectively, of the
portion of the hFSHR sequence coding for mature N-terminal
15 residues 1-349 (hFSHR-EC) lacking the endogenous signal
peptide and starting with Cys, and ending with Arg_{349}. Two
oligonucleotide primers, 5'-TTTTTGATCAGTGTCATCATCGGATCTGTC-3'
(SEQ ID NO: 3) and 5'-TTTTCTGCAGCTATCTGAGGATGTTGTACCCC-3' (SEQ
ID NO: 4), were designed to specifically amplify the DNA
20 sequence encoding mature N-terminal amino acid residues 1-349,
as well as incorporating a BclI restriction site at the 5' end
and a PstI and a TAG stop codon at the 3' end of the amplified
fragments.

PCR amplification was performed with these two
25 oligonucleotide primers for 25 complete PCR cycles with each
cycle involving a 30 second denaturation step at 99°C, a 30
second annealing step at 52°C, and a 1.25 minute
polymerization step at 75°C.

The fragment amplified from hFSHR cDNA was ligated
30 into baculovirus transfer vector pAcGP67-A (Catalogue #21220P
Pharmingen, San Diego, CA) at the BamHI and PstI cloning site
within a multiple cloning site immediately 3' from and in
frame with the *A. californica* glycoprotein gp67 signal
sequence. The resulting transfer vector pAcGP67-hFSHR-EC also
35 carries a strong baculovirus polyhedrin promoter, a flanking
polyhedrin region from the AcNPV virus as well as an *E. coli*
origin of replication and an ampicillin resistance gene for

plasmid amplification and selection in *E. coli* (Fig. 2).

　　　　As cloned into pAcGP67-A, the gene fragment encoding hFSHR-EC is expressed as a gp67 signal peptide fusion protein under the control of the strong baculovirus polyhedrin

5　promoter.   This creates a fusion between the N-terminus of the mature hFSHR-EC sequence and a signal sequence for an abundantly expressed baculovirus envelope surface glycoprotein (gp67) with the gp67 signal peptide serving to direct secretion of hFSHR-EC into the culture medium.   Figure 3 shows

10　that the ligation of the amplified fragment encoding hFSHR-EC into pAcGP67-A introduces three additional amino acid residues, Ala-Asp-Gln, between the presumed cleavage site of the gp67 signal peptide and the presumed mature hFSHR-EC open reading frame (beginning with Cys-His-His in Fig. 3).   Proper

15　vector construction was confirmed by nucleotide sequencing of the entire hFSHR-EC coding region with the constructed plasmid.

　　　　For the preparation of viral stocks, pAcGP67-FSHR-EC was transformed into *E. coli* strain MC1061, amplified and

20　purified by well-established methods (e.g., Gruenwald *et al.*, *Baculovirus Expression Vector System: Procedures and Methods Manual*, 2nd ed., Pharmigen, San Diego, CA, p. 38-44, 1993; QIAPREP silica purification membranes, Qiagen, Chatsworth, CA).   The purified transfer vector pAcGP67-hFSHR-EC was co-

25　transfected with BACULOGOLD DNA (Catalogue #21100D Pharmigen, San Diego, CA) into *Spodoptera frugiperda* Sf9 cells (American Type Culture Collection, Rockville, MD) using the calcium phosphate protocol (Gruenwald *et al.*, *supra*, p. 48-52). BACULOGOLD is a modified baculovirus DNA which contains a

30　lethal deletion and accordingly cannot encode for a viable virus by itself.   When co-transfected with a complementing transfer plasmid, such as pAcGP67-hFSHR-EC, carrying the essential gene lacking in BACULOGOLD, the lethal deletion is rescued and viable virus particles can be reconstituted inside

35　transfected insect cells.

Virus Purification, Amplification, and Verification

　　　　The virus generated in the co-transfection was

plaque purified (Gruenwald *et al.*, p. 51-52) and amplified
(Gruenwald *et al.*, p. 52-53) to generate virus particles for
further infections.

5   The structure of the plaque purified was verified by
PCR and Southern blotting. Viral DNA was prepared using virus
from the second round of amplification. Cell supernatants
from the amplification were filtered through a 0.2 μm filter
to remove cellular debris. 10 ml of the filtered supernatant
was centrifuged at 40,000 g for 30 minutes to pellet the
10 virus. The virus pellet was resuspended in 1 ml of TE (10 mM
Tris, 1 mM EDTA, pH 8.0). The resuspended virus was layered
onto a 5%/50% sucrose/PBS step gradient and centrifuged at
40,000 g for 30 minutes. The purified virus, which is located
between the two layers, was collected in a 1 ml volume and
15 diluted in 3 ml of TE. The diluted virus was centrifuged at
40,000 g for 30 minutes and the viral pellet was resuspended
in 200 μl of TE. The purified virus was treated with RNase A
(10 μg/ml) for 30 minutes at 37°C, then 0.5% SDS and
Proteinase K (10 μg/ml) were added and the mixture was
20 incubated for an additional 30 minutes at 37°C. The mixture
was phenol extracted, phenol:chloroform extracted, chloroform
extracted, and ethanol precipitated. The DNA pellet was
resuspended in 50 μl of TE.

1 μl of the viral DNA was used for PCR analysis
25 using primers located 5' and 3' of the hFSHR-EC sequence. PCR
was performed for 30 cycles of 1 minute at 99°C, 30 seconds at
56°C, and 1 minute at 75°C, followed by an extension step of 5
minutes at 75°C. When the PCR product was analyzed on an
agarose gel, a band of the expected size was obtained. When
30 the PCR generated DNA was digested with StuI, two bands
consistent with the expected structure of the recombinant
virus were obtained.

5 μg of the purified viral DNA was analyzed by
Southern blot (Ausubel *et al.*, eds., *Current Protocols in
35 Molecular Biology, supra*). The DNA was digested with either
BglII or PstI. The blots were probed with the PCR product
used in construction of the transfer vector that was labeled
by random oligo priming. The bands were of the expected sizes

and consistent with the predicted structure of the recombinant
virus.

Expression of hFSHR-EC in Insect Cells and Secretion into the
Culture Media

5   Viral stocks prepared from co-transfection as
described immediately above were used to infect HI-FIVE
Trichoplusia ni insect cells (Invitrogen, San Diego, CA) by
culturing in Grace's insect cell growth medium (Gibco BRL,
Cat. # 11605-011) supplemented with 10% heat inactivated fetal
10   bovine serum (Gibco BRL, Cat. # 16000-028, Life Technologies,
Gaithersburg, MD) for the heterologous production and
secretion of hFSHR-EC. The viral titer was confirmed by end-
point dilution and plaque assays following the procedures
described in the Pharmingen methods manual (Gruenwald *et al.*,
15   *supra*, p. 50-52) and in O'Reilly *et al.*, *Baculovirus
Expression Vectors, A Laboratory Manual*, W.H. Freeman and Co.,
New York, p. 155-157, 1992, both incorporated entirely herein
by reference. Infection of insect host cells in different
experiments was carried out at a multiplicity of infection
20   (MOI) of 2-10 and the media containing soluble hFSHR-EC was
collected at about 96 hour post infection (see also Gruenwald
et al., *supra*, p. 54-57).
          Culture media (3.5 ml) from $4x10^6$ insect cells
collected at 96 hours post-infection was serially diluted and
25   used directly in the binding assay described for supernatants
of CHO cell lysates in Example 1. The results of the binding
assay are shown in Figure 4 where it is clear that, with the
exception of the most concentrated or least diluted media
sample assayed (at a dilution factor of 0.2), the level of
30   specifically bound counts increase with increasing media
concentration until optimal media dilution of approximately
0.04 (25-fold dilution) is reached. A comparison of the
baculovirus/insect cell system (Figure 4) with the CHO cell
system (Figure 1 and Example 1) demonstrate the large increase
35   in soluble hFSH receptor expression in insect cells relative
to CHO cells (the sample used in the CHO cell system was
obtained from $1-5x10^7$ cells lysed into a final volume of 10 ml

buffer, whereas the culture media from the insect cell system
was derived from the 4x10⁶ cells at 96 hours post infection in
a media volume of 3.5 ml).

5   In a cold displacement/competitive binding assay of
   ¹²⁵I-hFSH to soluble hFSHR-EC, a stock solution of CHO-derived
   FSHR-EC was made by diluting the primary stock of CHO derived-
   hFSHR-EC (undiluted supernatant obtained from homogenized CHO
   cells for binding assays in Example 1) two-fold with
   sucrose/Hepes/Mg/Ca buffer containing 0.1% w/v BSA (SH-BSA
10 buffer). Another stock solution of insect cell-derived hFSHR-
   EC was also made by diluting the primary stock of insect cell
   hFSHR-EC, obtained from the culture media, 100-fold with SH-
   BSA buffer. Portions of these stock solutions (up to 80 µl)
   were combined with 10 µl (93,000 cpm, 1.1 nM) of ¹²⁵I-hFSH and
15 10 µl of serially diluted recombinant hFSH in SH-BSA buffer
   and sufficient SH-BSA buffer as make-up to reach 100 µl assay
   volume. As shown in Figure 5, increasing concentrations of
   non-radiolabeled hFSH compete with radiolabeled hFSH binding
   to hFSHR-EC and results in a net decrease of bound trace. It
20 is clear the hFSHR-EC derived from both CHO and insect cell
   systems exhibit similar binding characteristics.

          The level of hFSHR-EC expressed and secreted from
   insect cells was estimated to be approximately 0.2 mg/L
   culture media based on visualization on both silver-stained
25 and Coomassie blue-stained SDS-PAGE gels and by calculations
   using observed counts of ¹²⁵I-hFSH bound to soluble hFSHR-EC and
   the specific activity of the ¹²⁵I-hFSH (ca. 130 µCi/µg). Both
   CHO and insect cell-derived soluble hFSHR-EC do not require
   refolding in the presence of a mild denaturant, such
30 guanidine-HCl, as the soluble hFSHR-EC expressed in both
   systems is fully competent in high affinity binding to hFSH.

Example 3:
Scale-up of Secreted FSHR-EC Production

          Production conditions of approximately 3x10⁷ cells
35 seeded in each T175 flask containing 35 ml of Grace's insect
   cell medium (Gibco BRL) supplemented with 10% heat inactivated
   fetal bovine serum (Gibco BRL) were used to produce secreted

hFSH receptor in a 100 ml production scale-up culture of
infected *Trichoplusia ni* HI-FIVE insect cells. Western blot
and binding analysis were performed to compare media samples
from the current 100 ml scale-up production with a previous

5   smaller scale culture estimated to have ~ 0.2 mg/L of secreted
hFSH receptor in the culture medium based on binding data (see
Example 2 immediately above).

          Western blot analysis of the media samples from the
previous small scale batch and the current 100 ml batch showed

10 that a comparable level of secreted hFSH receptor on a per
unit volume basis was obtained for both batches.

          For the binding analysis (Figure 6), media samples
from either the previous batch or the new 100-ml batch were
serially diluted, mixed with 300,000 cpm of ^{125}I-hFSH (in the

15 presence or absence of 1 μM cold FSH for non-specific and
total counts, respectively), and incubated for 1 hour at 37°C.
Receptor-bound trace was precipitated by the addition of
polyethylene glycol. Bound counts were measured; and, as
shown in Figure 6, both sets of media samples showed similar

20 binding capacities at each dilution, whereas media from an
abundantly expressed glycoprotein not related to the
gonadotropin receptor in baculovirus/insect cells (negative
control) showed negligible specific binding. Therefore, by
both Western blotting and binding analysis, the 100-ml batch

25 shows comparable expression levels to those found for smaller-
scale production.

Affinity Purification of Soluble FSH Receptor

          Cross-linked recombinant human FSH (rhFSH)
heterodimer was used as ligand in affinity purification of

30 soluble FSH receptor because it is expected to generate a
stable ligand for making affinity resin that will undergo
repeated loading-elution steps without subunit dissociation.
Recombinant FSH (0.33 mg/ml in 50 mM MES, pH 5.5) was
crosslinked using a water soluble carbodiimide, 1-ethyl-3-(3'-

35 dimethylaminopropylcarbodiimide, hydrochloride), abbreviated
as EDAC, at a concentration of 25 mM which activates surface-
exposed carboxyl groups to form an amide bond with amino

groups of neighboring Lys residues. The crosslinked rhFSH was
desalted on reverse-phase (Vydac C4) and analyzed by SDS-PAGE.
The gel shows that crosslinking was successful since the
material (crosslinked rhFSH) does not migrate at a smaller
5   molecular weight (corresponding to separated subunits) after
treatment with a reducing agent as is the case for non-
crosslinked rhFSH. Approximately 8 mg of crosslinked rhFSH
was generated for use in ligand affinity purification of FSHR-
EC.

10   For coupling of r-hFSH to AffiGel-Hz resin (Bio Rad
Laboratories, Melville, NY), the kit (Cat. # 153-6060)
protocol was followed by adding 3 mL of 20 μM r-hFSH in H_2O to
0.3 mL of 0.117 M $NaIO_4$ combined with 0.3 mL of Bio Rad
coupling buffer (final conditions: 17 mM FSH, 83 mM $NaIO_4$, pH ~
15 5.7). The oxidized r-hFSH (3 mL) was desalted using the kit
desalting column (pre-equilibrated with 1X coupling buffer, pH
adjusted to 5.6), giving a 2.7 mL fraction of desalted protein
with an optical density (280 nm) of 0.425. Two mL of the
desalted, oxidized r-hFSH was combined with 2.5 mL of the
20 Affi-Gel resin which had been previously washed with 2x10 mL
of 1X coupling buffer (pH 5.7). After rotating the suspension
end-over-end for 12 hours at room temperature in a capped 15
mL conical tube, the optical density (280 nm) of the
supernatant had decreased to 0.09, suggesting a coupling yield
25 of 60-70%.

            To generate a 1 mL (bed volume) column, a portion
of the modified Affigel-Hz resin was added to a 0.5 cm (i.d)
column. The column was washed with 20 mL of 0.1 M Hepes, 0.1
M NaCl, pH 7.0 (HB) at 1 mL/minute, with 20 mL of 0.1 M Hepes,
30 0.5 M NaCl, pH 7.0 at 1 mL/minute, and then with 20 mL of HB
again at 1 mL/minute before loading culture media containing
hFSHR-EC. Media (10 ml) from the 100-ml batch of FSR-EC was
then loaded onto the column at ~ 0.2 ml/min at room
temperature, and the loaded column was washed with 100 mM
35 Hepes (pH 7.4)/100 mM NaCl and with 100 mM Hepes (pH 7.4)/500
mM NaCl, then eluted with 0.1 M HOAc (the washing/elution
portion of the chromatogram is shown in Figure 7). One ml
fractions were collected from the elution and then analyzed by

SDS-PAGE and Western blotting using polyclonal antibody X179.
Successful binding/elution of the receptor was observed with
the band corresponding to the soluble receptor being just
visible by Coumassie staining at between 42 and 60 kDa,
5   consistent with the MW expected for FSHR-EC. Thus, ten mls of
the 100-ml batch of FSHR-EC, subjected to affinity
chromatography using resin-bound rhFSH (non-crosslinked) as
ligand, resulted in a visible band on SDS-PAGE (Coumassie
staining) which appeared at the same position (between 42 and
10   60 kDa) as a band cross-reacting with anti-FSHR pAb (X179) in
Western blotting.

Example 4:
Demonstration of hFSH Antagonism By hFSHR-EC Using the
hFSHR-Y1 *in vitro* Assay
15   To assess whether hFSHR-EC in the culture medium
could antagonize hFSH action *in vitro*, the hFSHR-containing Y1
cell line (Y1-hFSHR) was used (Kelton *et al.*, *Mol. Cell.
Endocrin.* 89:141-151, 1992). These cells generate
progesterone in response to stimulation by hFSH. The
20   inhibitory effect of the culture medium containing hFSHR-EC on
Y1-hFSHR cells stimulated by a constant dose of hFSH was
determined.
          The culture medium used in this experiment was
prepared as follows. *Trichoplusia ni* HI-FIVE insect cells
25   were seeded in T175 flasks (~2.8x10^7 cells per flask)
containing 35 ml of Grace's insect medium (Gibco BRL, Cat. #
11605-011) supplemented with 10% heat inactivated fetal bovine
serum (Gibco BRL, Cat. # 16000-028) and then infected with
viral stocks of recombinant baculovirus at a multiplicity of
30   infection of five. Protein production was allowed to continue
ar 37°C for 4 days. Cells were transferred to 50 ml conical
flasks by gentle swirling and pipetting, and whole cells were
pelleted by centrifugation at 1000 rpm on an H6000A rotor (ca.
200xg) for 10 minutes at 4°C. The supernatant was decanted,
35   filtered through a 0.45 μm filter, and then centrifuged at
25,000 rpm on a Beckman L-70 ultracentrifuge for 20 minutes at
20°C using a SW-41 swinging bucket rotor.

For the Y1-hFSHR cell assay, 96-well plates were
seeded at a density of 2×10^4 Y1-hFSHR cells per well. The
cells were grown for three days in 200 μl of Y1 growth medium
(Ham's F-10, Gibco BRL, Cat. # I1550-027, supplemented with
15% horse serum and 2.5% fetal calf serum) at 37°C in a
humidified atmosphere containing 5% CO_2. The adherent cells at
70-90% confluency were washed once with 200 μl of assay medium
(Ham's F-10, 1% bovine serum albumen, 1% L-glutamine, 80 μg/ml
G418), and then 100 μl fresh assay medium was added to each
well followed by 100 μl aliquots of samples or standards
diluted in assay medium. For the antagonism assays, a
constant dose of hFSH (3×10^{-11} M), which in the absence of
antagonist gives 80% of maximal stimulation, was present in
each well, whereas the level of hFSHR-EC or control protein
(see below) was serially diluted in assay medium. The hFSH
plus hFSHR-EC mixture was either mixed at room temperature and
transferred to the Y1-hFSHR cells within one hour (Figure 8A),
or it was preincubated at 37°C for one hour before
transferring it to the Y1-hFSHR cells (Figure 8B).
Alternatively, to assess the effect of culture medium on an
independent pathway for adenylyl cyclase activation, a
constant dose of forskolin (1×10^{-5} M) was added to each well,
which stimulates high levels of progesterone production in Y1-
hFSHR cells. Cells supernatants were analyzed for
progesterone concentration using Serono Progesterone MAIA
Clone™ immunoassay kits. The concentration of hFSHR-EC shown
in the x-axis in Figures 8A and 8B was estimated based on ^{125}I-
hFSH binding data for the stock solution of hFSHR-EC culture
medium. Using these data, the concentration of hFSHR-EC in
the original culture medium was estimated to be 0.2 mg/L (4
nM, assuming a molecular weight of 50 kDa for hFSHR-EC. The
original culture medium was then diluted in assay medium to
give the concentration units shown in Figures 8A and 8B. The
highest estimated concentration of hFSHR-EC tested (8.9×10^{-11})
was made by diluting the original medium by a final dilution
factor (in the assay well) of 0.0223.

Several control experiments were also carried out,
some of which used culture medium from baculovirus/insect

cells expressing a secreted glycoprotein (the control protein)
other than hFSHR-EC. The cell culturing was conducted in
parallel for both the hFSHR-EC and control protein expression,
using the same conditions and buffers. The concentration of
5   this control protein was not accurately determined but is
probably much higher than that of hFSHR-EC. The objectives of
the control experiments were to assess: i) the effect of
culture medium alone on progesterone production in Y1-hFSHR
cells; ii) the effect of control protein culture medium in
10   hFSH-stimulated progesterone production in Y1-hFSHR cells; and
iii) the effect of hFSHR-EC or control protein-containing
baculovirus/insect cells culture media on production in Y1-
hFSHR cells stimulated by a pathway independent from the
gonadotropin receptor (forskolin pathway). The results of the
15   control experiments, summarized below (averages from at least
three data points, errors given as sample standard
deviations), can be compared with the data shown in Figures 8A
and 8B. First, treatment with FSHR-EC culture medium alone
resulted only in basal levels of progesterone production in
20   Y1-hFSHR cells (FSHR-EC alone at the highest concentration
used, i.e., 8.9×10^{-11} M or a 0.022-fold dilution of the original
culture medium, gave 4.9 ± 0.1 and 5.5 ± 0.2 ng/ml
progesterone in the non-preincubated and preincubated
experiments, respectively). Further, treatment with the
25   control protein culture medium alone (0.022-fold dilution from
stock) resulted in similar basal levels of progesterone ($4.7 \pm$
0.2 and 5.1 ± 0.3 ng/ml for non-preincubated and preincubated
experiments, respectively). Second, the control protein
culture medium (0.022-fold dilution from stock) did not
30   antagonize progesterone in Y1-hFSHR cells stimulated by 0.03
nM r-hFSH (28.2 ± 4.9 and 29.8 ± 3.5 ng/ml for non-
preincubated and preincubated experiments, respectively).
Third, progesterone production in Y1-hFSHR cells stimulated by
a constant dose (10 μM) of forskolin alone was 40.6 ± 4.5 and
35   31.2 ± 3.1 ng/ml for non-preincubated and preincubated
experiments, respectively. This stimulation by 10 μM
forskolin was not significantly reduced by treatment with
0.022-fold diluted FSHR-EC culture medium (37.3 ± 6.7 and 28.3

± 4.0 ng/ml, for non-preincubated and preincubated
experiments, respectively) or by 0.022-fold diluted control
protein culture medium (44.7 ± 3.8 and 23.0 ± 4 ng/ml for non-
preincubated and preincubated experiments, respectively).

5   In summary, Figures 8A and 8B and the results
described in the previous paragraph demonstrate that: i) FSH-
stimulated progesterone production can be completely inhibited
by hFSHR-EC in culture medium but is not affected by control
culture medium; ii) FSHR-EC or control protein alone in

10 culture medium do not stimulate progesterone production in Yl-
hFSHR cells; and iii) neither hFSHR-EC nor control protein
culture medium inhibit an independent pathway (forskolin
stimulation) of adenylyl cyclase activation. Finally, the
antagonistic effect of hFSHR-EC toward activation of hFSHR in

15 Y-1 cells does not require extensive preincubation of hFSHR-EC
with r-hFSH (compare Figures 8A and 8B).

    Having now fully described this invention, it will
be appreciated by those skilled in the art that the same can
be performed within a wide range of equivalent parameters,

20 concentrations, and conditions without departing from the
spirit and scope of the invention and without undue
experimentation.

    While this invention has been described in
connection with specific embodiments thereof, it will be

25 understood that it is capable of further modifications. This
application is intended to cover any variations, uses, or
adaptations of the inventions following, in general, the
principles of the invention and including such departures from
the present disclosure as come within known or customary

30 practice within the art to which the invention pertains and as
may be applied to the essential features hereinbefore set
forth as follows in the scope of the appended claims.

    All references cited herein, including journal
articles or abstracts, published or corresponding U.S. or

35 foreign patent applications, issued U.S. or foreign patents,
or any other references, are entirely incorporated by
reference herein, including all data, tables, figures, and
text presented in the cited references. Additionally, the

entire contents of the references cited within the references
cited herein are also entirely incorporated by reference.

Reference to known method steps, conventional
methods steps, known methods or conventional methods is not in
5   any way an admission that any aspect, description or
embodiment of the present invention is disclosed, taught or
suggested in the relevant art.

The foregoing description of the specific
embodiments will so fully reveal the general nature of the
10   invention that others can, by applying knowledge within the
skill of the art (including the contents of the references
cited herein), readily modify and/or adapt for various
applications such specific embodiments, without undue
experimentation, without departing from the general concept of
15   the present invention. Therefore, such adaptations and
modifications are intended to be within the meaning and range
of equivalents of the disclosed embodiments, based on the
teaching and guidance presented herein. It is to be
understood that the phraseology or terminology herein is for
20   the purpose of description and not of limitation, such that
the terminology or phraseology of the present specification is
to be interpreted by the skilled artisan in light of the
teachings and guidance presented herein, in combination with
the knowledge of one of ordinary skill in the art.

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(2) INFORMATION FOR SEQ ID NO:1:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 1316 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

   (ii) MOLECULE TYPE: cDNA

   (ix) FEATURE:
        (A) NAME/KEY: CDS
        (B) LOCATION: 138..1307

   (xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

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TTCGTAACAG TTTTGTAATA AAAAAACCTA TAAATATTCC GGATTATTCA TACCGTCCCA   120

CCATCGGGCG CGGATCT ATG CTA CTA GTA AAT CAG TCA CAC CAA GGC TTC   170
                   Met Leu Leu Val Asn Gln Ser His Gln Gly Phe
                    1   5   10

AAT AAG GAA CAC ACA AGC AAG ATG GTA AGC GCT ATT GTT TTA TAT GTG   218
Asn Lys Glu His Thr Ser Lys Met Val Ser Ala Ile Val Leu Tyr Val
            15   20   25

CTT TTG GCG GCG GCG GCG CAT TCT GCC TTT GCG GCG GAT CAG TGT CAT   266
Leu Leu Ala Ala Ala Ala His Ser Ala Phe Ala Ala Asp Gln Cys His
            30   35   40

CAT CGG ATC TGT CAC TGC TCT AAC AGG GTT TTT CTC TGC CAA GAG AGC   314
His Arg Ile Cys His Cys Ser Asn Arg Val Phe Leu Cys Gln Glu Ser
        45   50   55

AAG GTG ACA GAG ATT CCT TCT GAC CTC CCG AGG AAT GCC ATT GAA CTG   362
Lys Val Thr Glu Ile Pro Ser Asp Leu Pro Arg Asn Ala Ile Glu Leu
60   65   70   75

AGG TTT GTC CTC ACC AAG CTT CGA GTC ATC CAA AAA GGT GCA TTT TCA   410
Arg Phe Val Leu Thr Lys Leu Arg Val Ile Gln Lys Gly Ala Phe Ser
                80   85   90

GGA TTT GGG GAC CTG GAG AAA ATA GAG ATC TCT CAG AAT GAT GTC TTG   458
Gly Phe Gly Asp Leu Glu Lys Ile Glu Ile Ser Gln Asn Asp Val Leu
                95   100   105

GAG GTG ATA GAG GCA GAT GTG TTC TCC AAC CTT CCC AAA TTA CAT GAA   506
Glu Val Ile Glu Ala Asp Val Phe Ser Asn Leu Pro Lys Leu His Glu
            110   115   120

ATT AGA ATT GAA AAG GCC AAC AAC CTG CTC TAC ATC AAC CCT GAG GCC   554
Ile Arg Ile Glu Lys Ala Asn Asn Leu Leu Tyr Ile Asn Pro Glu Ala
        125   130   135

```
TTC CAG AAC CTT CCC AAC CTT CAA TAT CTG TTA ATA TCC AAC ACA GGT        602
Phe Gln Asn Leu Pro Asn Leu Gln Tyr Leu Leu Ile Ser Asn Thr Gly
140                 145                 150                 155

ATT AAG CAC CTT CCA GAT GTT CAC AAG ATT CAT TCT CTC CAA AAA GTT        650
Ile Lys His Leu Pro Asp Val His Lys Ile His Ser Leu Gln Lys Val
                160                 165                 170

TTA CTT GAC ATT CAA GAT AAC ATA AAC ATC CAC ACA ATT GAA AGA AAT        698
Leu Leu Asp Ile Gln Asp Asn Ile Asn Ile His Thr Ile Glu Arg Asn
            175                 180                 185

TCT TTC GTG GGG CTG AGC TTT GAA AGT GTG ATT CTA TGG CTG AAT AAG        746
Ser Phe Val Gly Leu Ser Phe Glu Ser Val Ile Leu Trp Leu Asn Lys
            190                 195                 200

AAT GGG ATT CAA GAA ATA CAC AAC TGT GCA TTC AAT GGA ACC CAA CTA        794
Asn Gly Ile Gln Glu Ile His Asn Cys Ala Phe Asn Gly Thr Gln Leu
    205                 210                 215

GAT GAG CTG AAT CTA AGC GAT AAT AAT AAT TTA GAA GAA TTG CCT AAT        842
Asp Glu Leu Asn Leu Ser Asp Asn Asn Asn Leu Glu Glu Leu Pro Asn
220                 225                 230                 235

GAT GTT TTC CAC GGA GCC TCT GGA CCA GTC ATT CTA GAT ATT TCA AGA        890
Asp Val Phe His Gly Ala Ser Gly Pro Val Ile Leu Asp Ile Ser Arg
            240                 245                 250

ACA AGG ATC CAT TCC CTG CCT AGC TAT GGC TTA GAA AAT CTT AAG AAG        938
Thr Arg Ile His Ser Leu Pro Ser Tyr Gly Leu Glu Asn Leu Lys Lys
            255                 260                 265

CTG AGG GCC AGG TCG ACT TAC AAC TTA AAA AAG CTG CCT ACT CTG GAA        986
Leu Arg Ala Arg Ser Thr Tyr Asn Leu Lys Lys Leu Pro Thr Leu Glu
            270                 275                 280

AAG CTT GTC GCC CTC ATG GAA GCC AGC CTC ACC TAT CCC AGC CAT TGC       1034
Lys Leu Val Ala Leu Met Glu Ala Ser Leu Thr Tyr Pro Ser His Cys
            285                 290                 295

TGT GCC TTT GCA AAC TGG AGA CGG CAA ATC TCT GAG CTT CAT CCA ATT       1082
Cys Ala Phe Ala Asn Trp Arg Arg Gln Ile Ser Glu Leu His Pro Ile
300                 305                 310                 315

TGC AAC AAA TCT ATT TTA AGG CAA GAA GTT GAT TAT ATG ACT CAG ACT       1130
Cys Asn Lys Ser Ile Leu Arg Gln Glu Val Asp Tyr Met Thr Gln Thr
            320                 325                 330

AGG GGT CAG AGA TCC TCT CTG GCA GAA GAC AAT GAG TCC AGC TAC AGC       1178
Arg Gly Gln Arg Ser Ser Leu Ala Glu Asp Asn Glu Ser Ser Tyr Ser
            335                 340                 345

AGA GGA TTT GAC ATG ACG TAC ACT GAG TTT GAC TAT GAC TTA TGC AAT       1226
Arg Gly Phe Asp Met Thr Tyr Thr Glu Phe Asp Tyr Asp Leu Cys Asn
            350                 355                 360

GAA GTG GTT GAC GTG ACC TGC TCC CCT AAG CCA GAT GCA TTC AAC CCA       1274
Glu Val Val Asp Val Thr Cys Ser Pro Lys Pro Asp Ala Phe Asn Pro
            365                 370                 375

TGT GAA GAT ATC ATG GGG TAC AAC ATC CTC AGA TAGCTGCAG                 1316
Cys Glu Asp Ile Met Gly Tyr Asn Ile Leu Arg
380                 385                 390
```

(2) INFORMATION FOR SEQ ID NO:2:

```
        (i) SEQUENCE CHARACTERISTICS:
            (A) LENGTH: 390 amino acids
            (B) TYPE: amino acid
            (D) TOPOLOGY: linear
       (ii) MOLECULE TYPE: peptide

       (xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Met Leu Leu Val Asn Gln Ser His Gln Gly Phe Asn Lys Glu His Thr
 1               5                  10                  15

Ser Lys Met Val Ser Ala Ile Val Leu Tyr Val Leu Leu Ala Ala Ala
            20                  25                  30

Ala His Ser Ala Phe Ala Ala Asp Gln Cys His His Arg Ile Cys His
        35                  40                  45

Cys Ser Asn Arg Val Phe Leu Cys Gln Glu Ser Lys Val Thr Glu Ile
    50                  55                  60

Pro Ser Asp Leu Pro Arg Asn Ala Ile Glu Leu Arg Phe Val Leu Thr
65                  70                  75                  80

Lys Leu Arg Val Ile Gln Lys Gly Ala Phe Ser Gly Phe Gly Asp Leu
                85                  90                  95

Glu Lys Ile Glu Ile Ser Gln Asn Asp Val Leu Glu Val Ile Glu Ala
            100                 105                 110

Asp Val Phe Ser Asn Leu Pro Lys Leu His Glu Ile Arg Ile Glu Lys
        115                 120                 125

Ala Asn Asn Leu Leu Tyr Ile Asn Pro Glu Ala Phe Gln Asn Leu Pro
    130                 135                 140

Asn Leu Gln Tyr Leu Leu Ile Ser Asn Thr Gly Ile Lys His Leu Pro
145                 150                 155                 160

Asp Val His Lys Ile His Ser Leu Gln Lys Val Leu Leu Asp Ile Gln
                165                 170                 175

Asp Asn Ile Asn Ile His Thr Ile Glu Arg Asn Ser Phe Val Gly Leu
            180                 185                 190

Ser Phe Glu Ser Val Ile Leu Trp Leu Asn Lys Asn Gly Ile Gln Glu
        195                 200                 205

Ile His Asn Cys Ala Phe Asn Gly Thr Gln Leu Asp Glu Leu Asn Leu
    210                 215                 220

Ser Asp Asn Asn Asn Leu Glu Glu Leu Pro Asn Asp Val Phe His Gly
225                 230                 235                 240

Ala Ser Gly Pro Val Ile Leu Asp Ile Ser Arg Thr Arg Ile His Ser
            245                 250                 255

Leu Pro Ser Tyr Gly Leu Glu Asn Leu Lys Lys Leu Arg Ala Arg Ser
            260                 265                 270

Thr Tyr Asn Leu Lys Lys Leu Pro Thr Leu Glu Lys Leu Val Ala Leu
        275                 280                 285

Met Glu Ala Ser Leu Thr Tyr Pro Ser His Cys Cys Ala Phe Ala Asn
    290                 295                 300

Trp Arg Arg Gln Ile Ser Glu Leu His Pro Ile Cys Asn Lys Ser Ile
305                 310                 315                 320
```

```
Leu Arg Gln Glu Val Asp Tyr Met Thr Gln Thr Arg Gly Gln Arg Ser
            325                 330             335

Ser Leu Ala Glu Asp Asn Glu Ser Ser Tyr Ser Arg Gly Phe Asp Met
            340                 345             350

Thr Tyr Thr Glu Phe Asp Tyr Asp Leu Cys Asn Glu Val Val Asp Val
            355                 360             365

Thr Cys Ser Pro Lys Pro Asp Ala Phe Asn Pro Cys Glu Asp Ile Met
    370             375             380

Gly Tyr Asn Ile Leu Arg
385             390
```

(2) INFORMATION FOR SEQ ID NO:3:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 30 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: oligonucleotide

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

```
TTTTTGATCA GTGTCATCAT CGGATCTGTC                                    30
```

(2) INFORMATION FOR SEQ ID NO:4:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 32 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: oligonucleotide

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

```
TTTTCTGCAG CTATCTGAGG ATGTTGTACC CC                                 32
```

(2) INFORMATION FOR SEQ ID NO:5:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 38 amino acids
        (B) TYPE: amino acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: peptide

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

```
Met Leu Leu Val Asn Gln Ser His Gln Gly Phe Asn Lys Glu His Thr
1               5               10              15

Ser Lys Met Val Ser Ala Ile Val Leu Tyr Val Leu Leu Ala Ala Ala
            20              25              30

Ala His Ser Ala Phe Ala
            35
```

```
Ala Ala Leu Cys Ser Ala
              20
```

(2) INFORMATION FOR SEQ ID NO:10:

     (i) SEQUENCE CHARACTERISTICS:
         (A) LENGTH: 16 amino acids
         (B) TYPE: amino acid
         (C) STRANDEDNESS: single
         (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: peptide

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

```
Met Phe Lys Phe Val Met Ile Cys Ala Val Leu Gly Leu Ala Val Ala
1               5                   10                  15
```

(2) INFORMATION FOR SEQ ID NO:11:

     (i) SEQUENCE CHARACTERISTICS:
         (A) LENGTH: 16 amino acids
         (B) TYPE: amino acid
         (C) STRANDEDNESS: single
         (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: peptide

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

```
Met Phe Lys Phe Val Met Ile Leu Ala Val Val Gly Val Ala Thr Ala
1               5                   10                  15
```

(2) INFORMATION FOR SEQ ID NO:12:

     (i) SEQUENCE CHARACTERISTICS:
         (A) LENGTH: 16 amino acids
         (B) TYPE: amino acid
         (C) STRANDEDNESS: single
         (D) TOPOLOGY: linear
    (ii) MOLECULE TYPE: peptide

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

```
Met Phe Lys Ile Leu Leu Val Cys Ser Leu Ala Ala Leu Val Ala Ala
1               5                   10                  15
```

(2) INFORMATION FOR SEQ ID NO:13:

     (i) SEQUENCE CHARACTERISTICS:
         (A) LENGTH: 11 amino acids
         (B) TYPE: amino acid
         (C) STRANDEDNESS: single
         (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: peptide

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

```
Met Phe Lys Ile Leu Leu Val Cys Ala Leu Val
1               5                   10
```

(2) INFORMATION FOR SEQ ID NO:14:

(2) INFORMATION FOR SEQ ID NO:6:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 18 amino acids
        (B) TYPE: amino acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: peptide

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

    Met Thr Ile Leu Cys Trp Leu Ala Leu Leu Ser Thr Leu Thr Ala Val
    1   5   10   15

    Asn Ala

(2) INFORMATION FOR SEQ ID NO:7:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 24 amino acids
        (B) TYPE: amino acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: peptide

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

    Met Val Ser Ala Ile Val Leu Tyr Val Leu Leu Ala Ala Ala Ala His
    1   5   10   15

    Ser Ala Phe Ala Ala Glu His Cys
           20

(2) INFORMATION FOR SEQ ID NO:8:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 19 amino acids
        (B) TYPE: amino acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: peptide

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

    Met Tyr Lys Leu Thr Val Phe Leu Met Phe Ile Ala Phe Val Ile Ile
    1   5   10   15

    Ala Glu Ala

(2) INFORMATION FOR SEQ ID NO:9:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 22 amino acids
        (B) TYPE: amino acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: peptide

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

    Met Val Gln Arg Cys Leu Val Val Ala Leu Leu Val Val Val Val Ala
    1   5   10   15

```
    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 17 amino acids
        (B) TYPE: amino acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

   (ii) MOLECULE TYPE: peptide

   (xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

   Met Phe Leu Ala Lys Ser Ile Val Cys Leu Ala Leu Leu Ala Val Ala
   1               5               10              15

   Asn
```

(2) INFORMATION FOR SEQ ID NO:15:

```
    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 20 amino acids
        (B) TYPE: amino acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

   (ii) MOLECULE TYPE: peptide

   (xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

   Met Ser Thr Phe Ala Phe Leu Leu Leu Cys Ile Gln Ala Cys Leu Ile
   1               5               10              15

   Gln Asn Val Tyr
                20
```

(2) INFORMATION FOR SEQ ID NO:16:

```
    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 21 amino acids
        (B) TYPE: amino acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

   (ii) MOLECULE TYPE: peptide


   (xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

   Met Asn Phe Ser Arg Ile Phe Phe Phe Val Phe Ala Leu Val Leu Ala
   1               5               10              15

   Leu Ser Thr Ala Ser
                20
```

(2) INFORMATION FOR SEQ ID NO:17:

```
    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 21 amino acids
        (B) TYPE: amino acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

   (ii) MOLECULE TYPE: peptide


   (xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:
```

```
Met Asn Phe Ser Arg Ile Phe Phe Phe Val Phe Ala Leu Val Leu Ala
1               5                   10                  15

Leu Ser Thr Val Ser
            20
```

(2) INFORMATION FOR SEQ ID NO:18:

   (i) SEQUENCE CHARACTERISTICS:
     (A) LENGTH: 26 amino acids
     (B) TYPE: amino acid
     (C) STRANDEDNESS: single
     (D) TOPOLOGY: linear

   (ii) MOLECULE TYPE: peptide

   (xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

```
Met Ala Met Ile Pro Ser Ile Ser Lys Leu Leu Phe Val Ala Ile Cys
1               5                   10                  15

Leu Phe Val Tyr Met Gly Leu Ser Phe Gly
            20                  25
```

WHAT IS CLAIMED IS:

1. A recombinant baculovirus transfer vector,
comprising a DNA segment encoding a baculovirus signal peptide
joined to a DNA segment encoding a protein essentially
corresponding to the extracellular domain fragment of a
gonadotropin receptor translationally in frame with said
signal peptide and operably linked to a baculovirus promoter,
said protein being capable of binding the hormone ligand of
said receptor.

2. A recombinant baculovirus transfer vector
according to claim 1, wherein said extracellular domain of
said gonadotropin receptor is hFSHR-EC.

3. A recombinant baculovirus transfer vector
according to claim 1, wherein said extracellular domain of
said gonadotropin receptor is hLH/CGR-EC.

4. A recombinant baculovirus transfer vector
according to claim 2, wherein said hFSHR-EC has the amino acid
sequence essentially corresponding to amino acid residues 39-
390 of SEQ ID NO: 2.

5. A recombinant baculovirus transfer vector
according to claim 1, wherein said baculovirus signal peptide
is the gp67 signal peptide of *Autographa californica*.

6. A recombinant baculovirus transfer vector
according to claim 5, wherein said gp67 signal peptide has the
amino acid sequence of SEQ ID NO: 5.

7. A method of producing secreted gonadotropin
receptors in soluble hormone-binding form, comprising the
steps of:

    constructing the recombinant transfer vector of
    claim 1;

    generating recombinant baculovirus by co-
    transfecting the recombinant transfer vector with
    baculovirus DNA or transfecting with the recombinant
    transfer vector into first insect cells;

    infecting second insect cells with the recombinant
    baculovirus and culturing the infected insect cells in a
    culture medium to express and secrete soluble

gonadotropin receptor capable of binding hormone ligand
of said receptor; and

        collecting the culture medium and purifying the
soluble gonadotropin receptor.

        8. A method according to claim 7, wherein the
second insect cells infected with recombinant baculovirus are
HI-FIVE *Trichoplusia ni* insect cells.

        9. Insect cells infected with recombinant
baculovirus, wherein said insect cells contain a DNA segment
encoding a baculovirus signal peptide joined to a DNA segment
encoding a protein essentially corresponding to the
extracellular domain fragment of a gonadotropin receptor
translationally in frame with the signal peptide and operably
linked to a baculovirus promoter, said protein being expressed
and secreted from said insect cells is capable of binding the
hormone ligand of said receptor.

        10. The insect cells according to claim 9, wherein
said insect cells are *Trichoplusia ni* insect cells.

        11. The insect cells according to claim 9, wherein
said extracellular domain of said gonadotropin receptor is
hFSHR-EC.

        12. The insect cells according to claim 9, wherein
said extracellular domain of said gonadotropin receptor is
hLH/CGR-EC.

        13. The insect cells according to claim 11, wherein
said hFSHR-EC has the amino acid sequence essentially
corresponding to amino acid residues 39-390 of SEQ ID NO: 2.

        14. The insect cells according to claim 9, wherein
said baculovirus signal peptide is the gp67 signal peptide of
Autographa californica.

        15. The insect cells according to claim 14, wherein
said gp67 signal peptide has the amino acid sequence of SEQ ID
NO: 5.

        16. A method of producing the insect cells of claim
9, comprising the steps of:

        infecting insect cells with recombinant baculovirus;
    and

        culturing the infected cells in culture medium.

17.   A method of purifying soluble gonadotropin
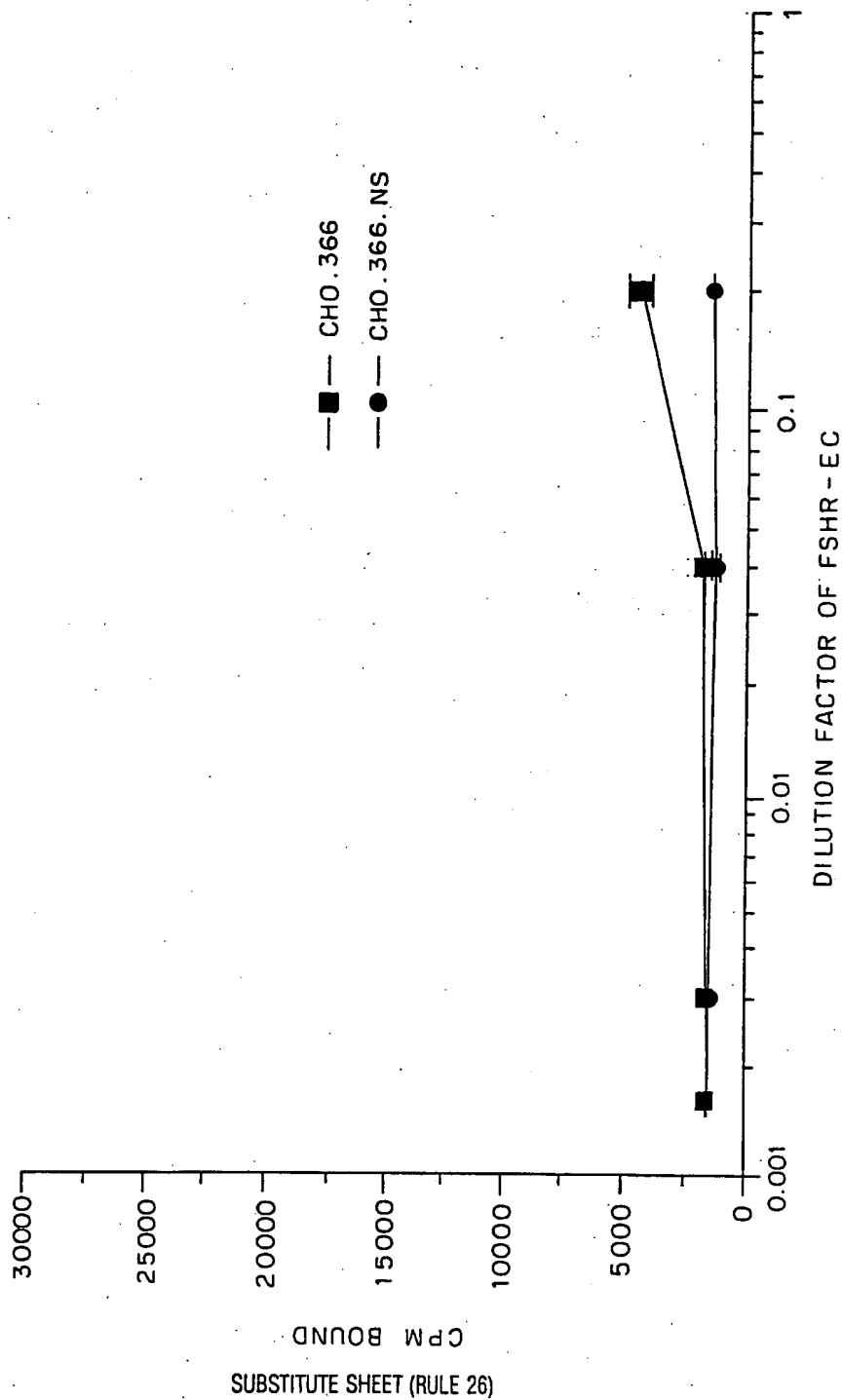receptors from insect cell culture media, comprising the steps
of:

        providing an affinity chromatography column having
    cross-linked gonadotropin glycoprotein hormones as
    ligands;

        binding soluble gonadotropin receptors to the
    hormone ligands;

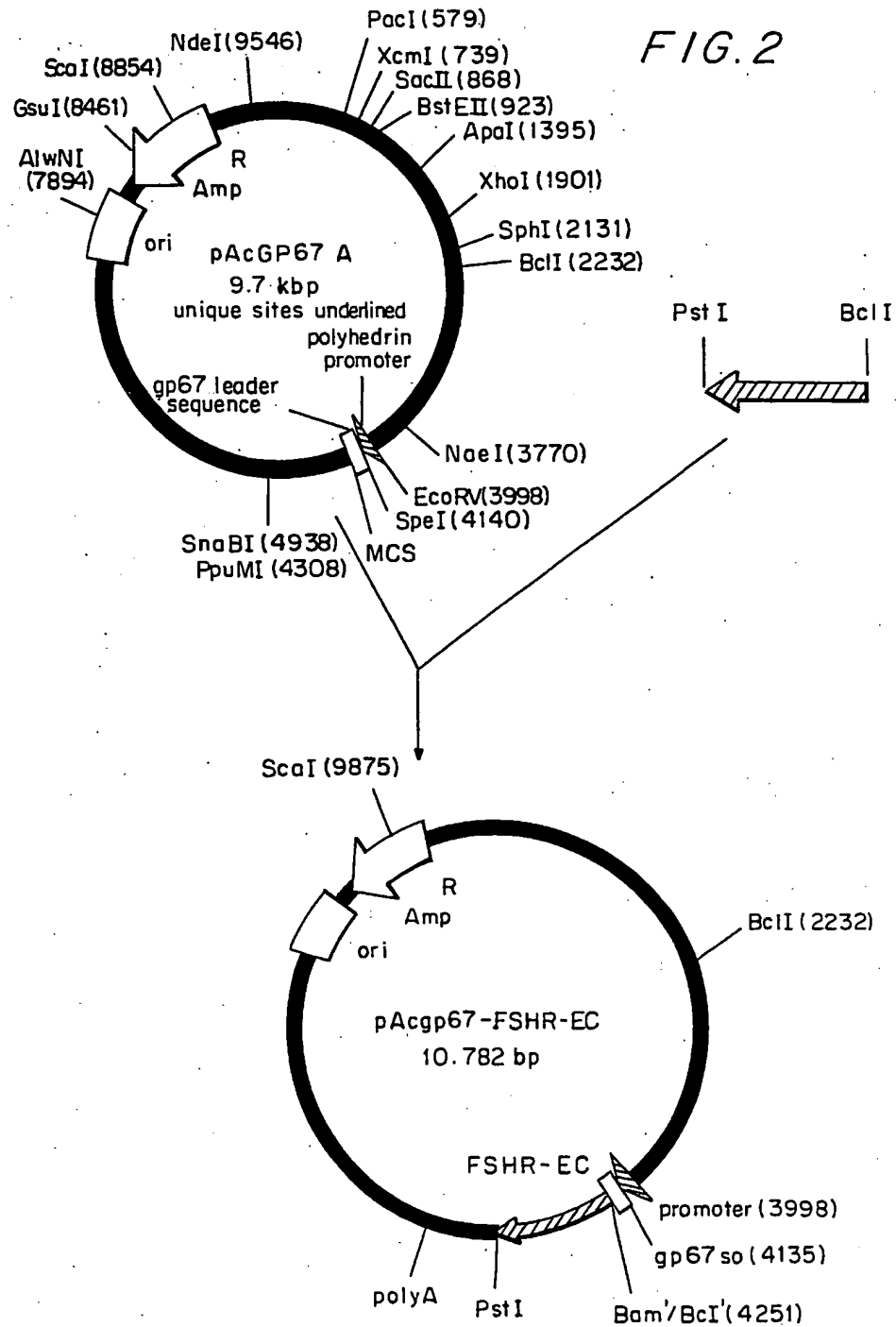   eluting off the soluble gonadotropin receptor;
    and

        collecting fractions containing the eluted soluble
    gonadotropin receptor.

F/G. 1

FIG.2

SUBSTITUTE SHEET (RULE 26)

F/G.34

```
        polyhedrin promoter
    1   GATATCATGGAGGATAATTAAAAATGATAACCATCTCGCAAATAAATAAGTATTTTACTGTTTTCGTAACAGTTTGTAATAAAAACCT

        polyhedrin'                                            gp67 signal peptide
   90   ATAAAT ATT CCG GAT TAT TCA TAC CGT CCC ACC ATC GGG CGC GGA TCT ATG CTA CTA GTA AAT
                                                              1► Met Leu Leu Val Asn

  153   CAG TCA CAC CAA GGC TTC AAT AAG GAA CAC ACA AGC AAG ATG GTA AGC GCT ATT GTT
    6►  Gln Ser His Gln Gly Phe Asn Lys Glu His Thr Ser Lys Met Val Ser Ala Ile Val

  210   TTA TAT GTG CTT TTG GCG GCG GCG CAT TCT GCC TTT GCG
   25►  Leu Tyr Val Leu Leu Ala Ala Ala His Ser Ala Phe Ala ▼

        BamHI/BclI  -1 mature FSHR (putative)
  252   GCG GAT CAG TGT CAT CAT CGG ATC TGT CAC TGC TCT AAC AGG GTT CTC TTT TGC CAA GAG AGC AAG
   39►  Ala Asp Gln Cys His His Arg Ile Cys His Cys Ser Asn Arg Val Phe Leu Cys Gln Glu Ser Lys

  318   GTG ACA GAG ATT CCT TCT GAC CTC CCG AGG AAT GCC ATT GAA CTG AGG TTT GTC CTC ACC AAG CTT
   61►  Val Thr Glu Ile Pro Ser Asp Leu Pro Arg Asn Ala Ile Glu Leu Arg Phe Val Leu Thr Lys Leu

  384   CGA GTC ATC CAA AAA GGT GCA TTT TCA GGA TTT GGG GAC CTG GAG ATA ATA GAG ATC TCT CAG AAT
   83►  Arg Val Ile Gln Lys Gly Ala Phe Ser Gly Phe Gly Asp Leu Glu Ile Ile Glu Ile Ser Gln Asn

  450   GAT GTC TTG GAG GTG ATA ATA GAG GCA GAT CCC AAA CTT TTA CAT GAA ATT AGA ATT
  105►  Asp Val Leu Glu Val Ile Ile Glu Ala Asp Pro Lys Leu His Glu Ile Arg Ile

  516   GAA AAG GCC AAC AAC CTG TAC CTC AAC ATC CCT GAG GCC TTC CAG GCC CTT CCC AAC CTT CAA TAT
  127►  Glu Lys Ala Asn Asn Leu Tyr Leu Asn Ile Pro Glu Ala Phe Gln Ala Leu Pro Asn Leu Gln Tyr

  582   CTG TTA ATA TCC AAC ACA GGT ATT AAG CCA GAT GTT CAC ATT CAT CAC AAG ATT CAT CTC CAA AAA
  149►  Leu Leu Ile Ser Asn Thr Gly Ile Lys Pro Asp Val His Ile His His Lys Ile His Leu Gln Lys

  648   GTT CTT GAC ATT CAA GAT AAC ATC ACA ATA AAC ATC AAC GAA AGA AAT TCT TTC GTG GGG CTG
  171►  Val Leu Asp Ile Gln Asp Asn Ile Thr Ile Asn Ile Asn Glu Arg Asn Ser Phe Val Gly Leu
```
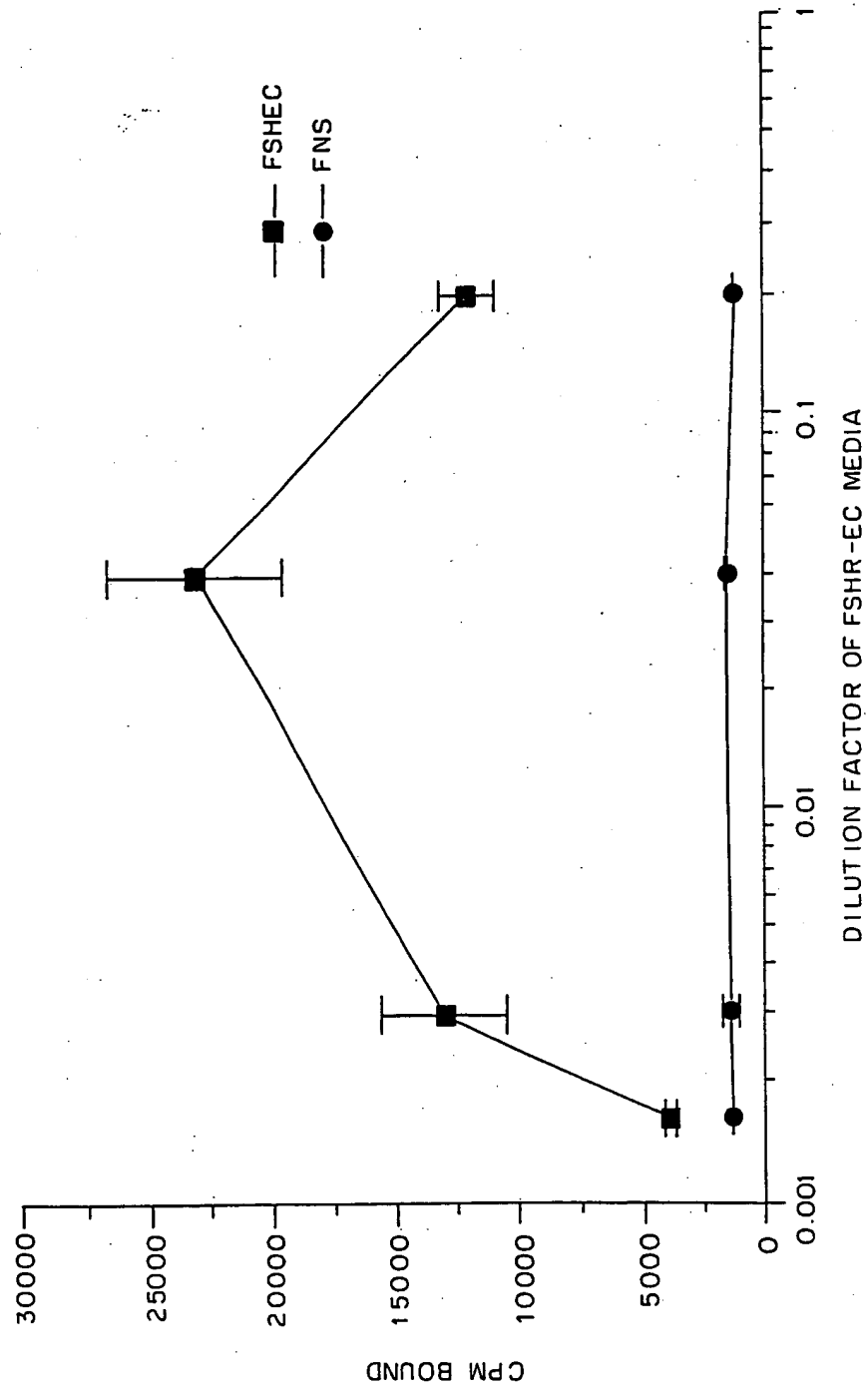
FIG. 3B

```
714   AGC TTT GAA AGT GTG ATT CTA TGG CTG AAT AAG AAT GGG ATT CAA GAA ATA CAC AAC TGT GCA TTC
193 ▶ Ser Phe Glu Ser Val Ile Leu Trp Leu Asn Lys Asn Gly Ile Gln Glu Ile His Asn Cys Ala Phe

780   AAT GGA ACC CAA CTA GAT GAG CTG AAT CTA AGC GAT GAT AAT AAT TTA GAA TTG GAA CCT AAT GAT
215 ▶ Asn Gly Thr Gln Leu Asp Glu Leu Asn Leu Ser Asp Asp Asn Asn Leu Glu Leu Glu Pro Asn Asp

846   GTT TTC CAC GGA GCC TCT ATT GTC CTA GAT ATT TCA GAT ATT CTA AGG ACA ATC CAT TCC CTG CCT
237 ▶ Val Phe His Gly Ala Ser Ile Val Leu Asp Ile Ser Asp Ile Leu Arg Thr Ile His Ser Leu Pro

912   AGC TAT GGC TTA GAA AAT CTT AAG AAG CTG AAG GCC AGG TCG ACT TAC AAC TTA AAA AAG CTG CCT
259 ▶ Ser Tyr Gly Leu Glu Asn Leu Lys Lys Leu Lys Ala Arg Ser Thr Tyr Asn Leu Lys Lys Leu Pro

978   ACT CTG GAA AAG CTT GTC GCC GAA GCC CTC ATG GAA ACC TAT CCC AGC CAT TGC TGT GCC TTT
281 ▶ Thr Leu Glu Lys Leu Val Ala Glu Ala Leu Met Glu Thr Tyr Pro Ser His Cys Cys Ala Phe

1044  GCA AAC TGG AGA CGG CAA ATC TCT GAG CTT CAT CCA ATT TGC AAC AAA TCT ATT TTA AGG CAA GAA
303 ▶ Ala Asn Trp Arg Arg Gln Ile Ser Glu Leu His Pro Ile Cys Asn Lys Ser Ile Leu Arg Gln Glu

1110  GTT GAT TAT ATG ACT CAG AGG GGT ACT TCC AGA GAT GCA GAA AAT GAC AAT GAG TCC AGC TAC
325 ▶ Val Asp Tyr Met Thr Gln Arg Gly Thr Ser Arg Asp Ala Glu Asn Asp Asn Glu Ser Ser Tyr

1176  AGC AGA GGA TTT GAC ACG ATG ACG TAC ACT GAG TTT GAC TAT GAC TTA TGC AAT GAA GTG GTT GAC GTG
347 ▶ Ser Arg Gly Phe Asp Thr Met Thr Tyr Thr Glu Phe Asp Tyr Asp Leu Cys Asn Glu Val Val Asp Val

1242  ACC TGC TCC CCT AAG CCA TTC AAC CCA GAT GCA TGT GAT ATC ATG GGG TAC ATC AAC ATC CTC AGA
369 ▶ Thr Cys Ser Pro Lys Pro Phe Asn Pro Asp Ala Cys Asp Ile Met Gly Tyr Ile Asn Ile Leu Arg

                                       (1311) PstI
1308  TAG CTG CAG
391 ▶ ....
```
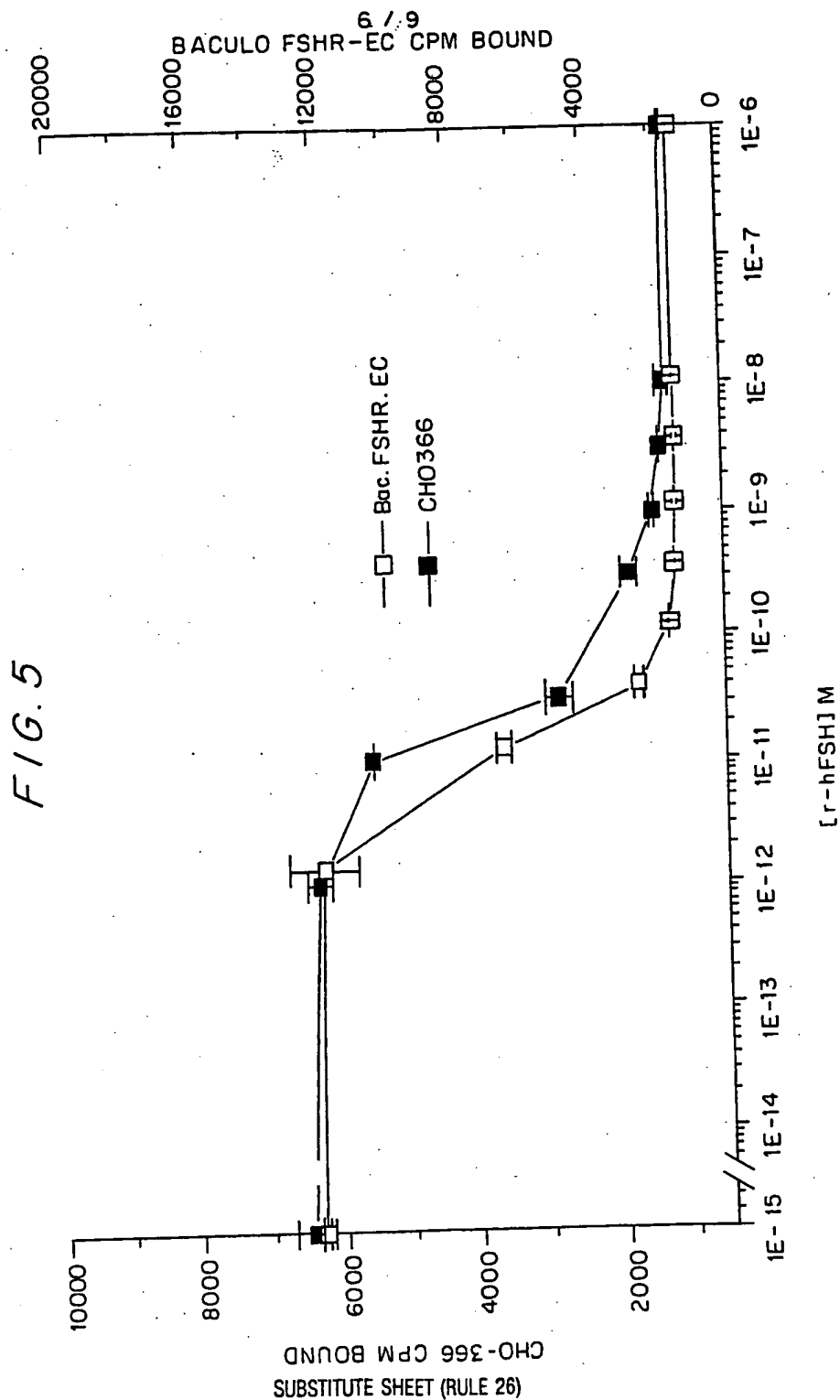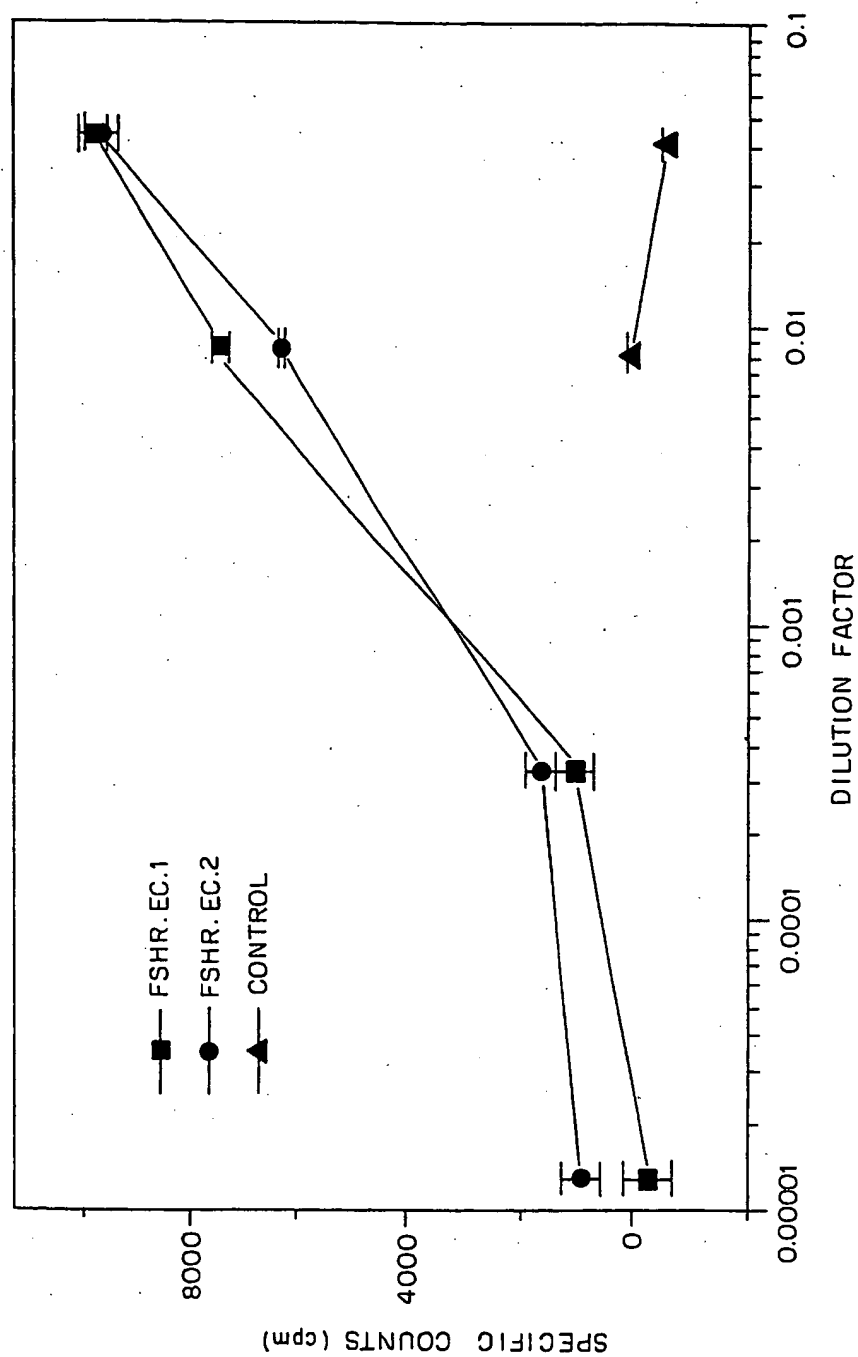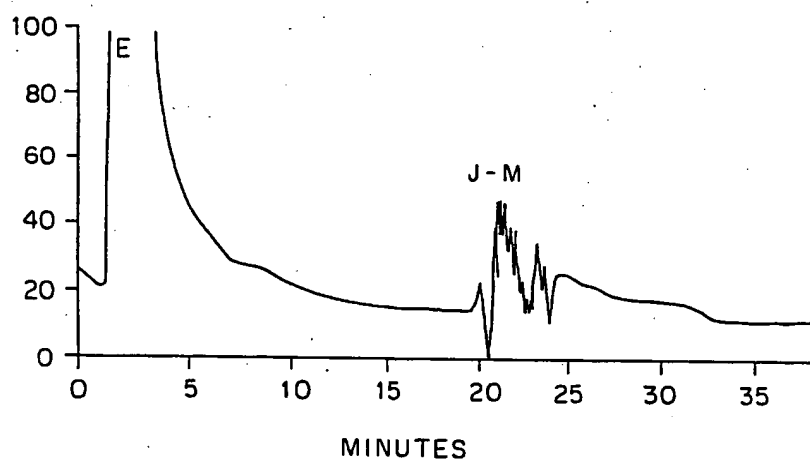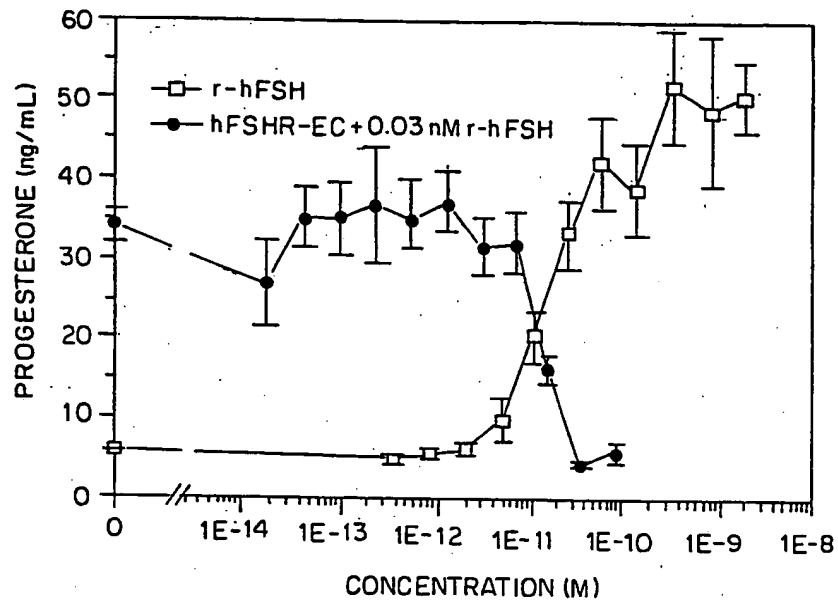
FIG. 4

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FIG.5

FIG.6

FIG. 7

F I G.8A

F I G.8B

INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/86 C12N15/12 C07K14/72 C12N5/06

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	ENDOCRINOLOGY, vol. 134, no. 2, February 1994, pages 549-554, XP000608927 G.S.SEETHARAMAIAH ET AL.: "A recombinant extracellular domain of the thyrotropin (TSH) receptor binds TSH in the absence of membranes" see paragraph bridging pages 549 and 550 --- -/--	1-16

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
6 November 1996	1 5. 11. 96

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Cupido, M

Form PCT/ISA/210 (second sheet) (July 1992)

	national Application No
	PCT/IB 96/00709

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	JOURNAL OF MOLECULAR ENDOCRINOLOGY, vol. 10, no. 2, April 1993, pages 127-143, XP000608924 G.C.HUANG ET AL.: "The thyrotrophin hormone receptor of Graves' disease: overexpression of the extracellular domain in insect cells using recombinant baculovirus, immunoaffinity purification and analysis of autoantibody binding" see page 130 - page 131 ---	1-17
A	EP,A,0 505 207 (THE WELLCOME FOUNDATION LTD;NATURAL ENVIRONMENT RESEARCH COUNCIL) 23 September 1992 see page 11; figure 14 -----	5,6,14, 15

1

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
EP-A-0505207	23-09-92	AU-A-	1549692	21-10-92
		BR-A-	9205803	28-06-94
		CA-A-	2106587	23-09-92
		WO-A-	9216637	01-10-92
		HU-A-	66757	28-12-94
		JP-T-	6510660	01-12-94
		OA-A-	9816	15-04-94